

THE CHEMISTRY OF  
LEONTICE LEONTOPETALUM, LINN  
AND  
FURTHER STUDIES ON THE  
SAPONINS OF  
CAULOPHYLLUM THALICTROIDES

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A THESIS

submitted to

The University of Glasgow

by

John McShefferty

in fulfilment of the  
requirements for the degree of  
DOCTOR of PHILOSOPHY

March, 1957.

The School of Pharmacy,  
The Royal College of  
Science and Technology,  
Glasgow.

The author wishes to thank Professor J.P.Todd for his continued interest and encouragement, and Dr.J.B. Stenlake, under whose direct guidance this work was carried out, for his useful criticisms and wealth of helpful suggestions.

Thanks are also due to the Pharmaceutical Society for an Educational Grant, and to Mr.D.J.Duff and Dr.W.M.Cumming of British Dyewoods Co. Ltd., for facilities and help with the large scale extraction.



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PART I

THE CHEMISTRY OF

LEONTICE LEONTOPETALUM, LINN.

July, 1901, a communication received from Dr. Harrison, Medical Director of the Ontario Hospital for the Insane and Nervous Disorders, London, Ont., on the use of a local plant in the treatment of

Dr. A. S. Haugli, the Senior Physician at the

has discovered, during the course of the study in Iceland, that this plant was a recognized

the plant is a small, low, bushy plant with a few leaves and small flowers. It is a native of the mountains and is found in the same places as the other plants of the same family.

## HISTORICAL INTRODUCTION

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In July, 1951, a communication<sup>(1)</sup> received from Dr.W. M.Ford-Robertson, Medical Director of the Lebanon Hospital for Mental and Nervous Disorders, Asfuriyeh, near Beirut, reported the use of a local plant in the treatment of epilepsy. Dr.A.S.Manugian, the Senior Physician at the Hospital had discovered, during the course of his studies on epilepsy in Lebanon, that this plant has a reputation as a native remedy. The plant, known locally as "Dough of the Earth", has succulent stems and leaves and tuberous roots which resemble soft watery potatoes. The bitter and poisonous "juice" used in the treatment<sup>(2)</sup> is prepared as required from freshly collected tubers which are dug up after the aerial parts have died down, cut into small pieces and pounded in a mortar with the minimum amount of water. The "juice" so obtained is given in doses of one teaspoonful thrice daily, and this treatment is continued on an average for three days. During this time the patient becomes very ill, and is more or less in status epilepticus, having convulsions which recur every two or three minutes. At this stage treatment is continued with large doses, (glassfuls), of a second aqueous extract prepared from the marc which remains after the expression of the "juice". This extract is administered frequently during each day, often over a period of several months. According to the native prescribers, the convulsive action of the drug ceases with

the administration of this second extract, but Dr.Manugian believes that this is not an essential part of the treatment.

It is understood that several confirmed epileptics have (apparently) been cured by the drug, though the treatment caused serious illness for some three to seven days, due no doubt to the toxic nature of the drug. One case, that of a thirteen year old girl with severe grand and petit mal epilepsy, has been reported in which complete remission from fits was observed for a period of six weeks following treatment. The patient, however, was seriously ill during the period of treatment.

When the drug was first received in this department, its botanical name was unknown, but in July, 1952, Mr.C.W. Highwood of the British Council in Beirut, suggested<sup>(3)</sup> that the plant was probably Leontice leontopetalum, Linn., a genus of the Berberidaceae. In order to confirm this identification the plant was examined in its natural habitat by Mr.P.F.Nelson of this department. Specimens of the plant in the flowering and fruiting condition were collected, preserved and finally compared with authentic specimens at the British Museum of Natural History, London, when the plant was in fact shown to be Leontice leontopetalum, Linn<sup>(4)</sup>.

The plant is a hardy perennial herb, growing to a height of one, to one and a half feet and has large compound leaves. The name Leontice is an abridgement of its ancient name

Leontopetalon and is derived from  $\lambda\epsilon\omega\nu$  (a lion) and  $\pi\epsilon\tau\alpha\lambda\omicron\nu$  (a leaf) because of the resemblance of the leaf to the print of a lion's foot<sup>(5)</sup>. The flower head is a compound inflorescence consisting of a panicle of yellow flowers, which produce purplish bladdery fruits. The large root-tubers are irregularly circular to oval in shape, 7 to 23 cm. in diameter, and covered with a thick brown cork and small protuberances, from which fine white rootlets arise. The part of the plant used as a drug has been variously described as a root<sup>(6)</sup>, tuberous root<sup>(5)(7)(8)</sup>, corm<sup>(6)</sup> tuberous stem base<sup>(9)</sup> or tuberous rhizome<sup>(10)</sup>. A more recent examination, however, indicates that it is an intercalary root-tuber<sup>(11)</sup>.

The plant grows in mountainous regions and abounds as a weed in many wheatfields in Central Lebanon<sup>(6)</sup>. It has also been reported widely throughout the Eastern Mediterranean, <sup>(5)</sup>, being observed in Italy<sup>(7)(8)(12)</sup>, Greece<sup>(7)(12)</sup>, Turkey<sup>(7)(8)(10)(12)(13)</sup>, Armenia<sup>(7)</sup>, Syria<sup>(6)(7)(14)</sup>, Israel<sup>(6)(7)(14)</sup>, Jordan<sup>(6)</sup>, Iraq<sup>(7)</sup>, north-eastern parts of Egypt<sup>(6)</sup> and on the island of Cyprus<sup>(7)</sup>.

The material examined was collected in the Bekaa plain in Lebanon, in the late Spring after the aerial parts had died down. The earliest specimens were found, on arrival in this country to be covered in mould, due to incomplete drying. Later, more satisfactory arrangements for collection



were made, and the freshly collected tubers were washed, sliced and dried rapidly in the sun. The dried drug consisted of irregularly shaped slices 0.5 to 1.0 cm. thick with a hard brittle texture. Externally there was a thick brown cork, the remainder of the drug being a yellowish-brown colour.

## HISTORY OF LEONTICE SPECIES

The Greeks used two species of Leontice as medicines and Gunther<sup>(16)</sup>, in his work, the Greek Herbal of Dioscorides, identified one, Leontopetalon, as L. chrysogonum, and the other Chrusogonum, as L. leontopetalum.

Leontopetalon (L. chrysogonum) was described as a small shrub, with purple flowers and black roots having excrescences like knobs. The leaves, from a drawing of the plant in the work, do bear some resemblance to the print of a lion's foot, hence the Greek name for the drug. It was apparent from the description and drawing of the plant that this plant was different from L. leontopetalum. Leontopetalon was used by the Greeks as a snake-bite remedy and as a remedy for sciatica.

Chrusogonum (L. leontopetalum). Comparison of the drawing of this plant with L. leontopetalum showed a very marked difference in the two plants. Gunther, however, mentions some doubt as to the genuineness of the drawings shown in the herbal. Moreover, the colour of the inner part of the root of Chrusogonum was also described as strongly red, whereas that of genuine L. leontopetalum is bright yellow. Thus some doubt exists as to the genuineness of Gunther's identification of Chrusogonum as L. leontopetalum. Chrusogonum was used by the Greeks for

the "bitings of the shrew mouse".

Loudon, in his work *The Encyclopaedia of Plants*<sup>(5)</sup>, mentions that *Leontopetalon* was first introduced into Britain in 1597. Lovell<sup>(15)</sup> in his *Compleat Herball* of 1665, mentions this plant, also known as *Rapesium* or *Papaverculum*, and states that it was found among corn in Italy and flowered in the winter. The roots were taken in wine as a snakebite antidote, were used in the treatment of sciatica, and for healing "old filthy ulcers". He also describes the use of the powdered roots by the natives of Aleppo (in Northern Syria), as a soap substitute.

Various brief botanical descriptions of Leontice leontopetalum have appeared at various times in the literature<sup>(5)(6)(7)(8)(9)(10)(14)(17)(18)(19)(20)</sup>. The root-tubers of Leontice leontopetalum have been used as a soap substitute<sup>(13)(14)(21)(22)</sup>, a snakebite antidote<sup>(14)</sup>, a corrective for overdoses of opium<sup>(13)</sup> and as a native remedy for epilepsy in the Holy Land<sup>(1)(2)(6)(8)(14)</sup>. The plant has never been investigated chemically and the present work was undertaken to isolate the chemical constituents of L. leontopetalum, and to identify the constituents responsible for the action of the drug when used in epilepsy.

## CHEMICAL CONSTITUENTS OF LEONTICE SPECIES

Few investigations of the chemical constituents of plants of the genus Leontice, family Berberidaceae, have been reported. The genus originally known as Leontice, has now been sub-divided into the two new genera, of Leontice and Caulophyllum. A drug bearing the name Caulophyllum, official in the British Pharmaceutical Codex of 1934<sup>(23)</sup> was derived from the plant Caulophyllum thalictroides, formerly known as Leontice thalicttrum, which is found in North America and Japan. It was first examined chemically by Mayer<sup>(24)</sup> in 1863, who found evidence of a saponaceous principle and an alkaloid. In the following year, Ebert<sup>(25)</sup> also reported these "principles", but failed to isolate them from the plant. The alkaloid was first obtained as an amorphous solid by Lloyd<sup>(26)(27)</sup>, who called it "caulophylline", but did not determine its composition. He also isolated the crystalline glycoside, leontin. The pure crystalline alkaloid was finally obtained by Power and Salway<sup>(28)</sup>, in 1913, and shown to be methylcytisine. They also isolated two crystalline saponins, caulosaponin and caulophyllosaponin, which are discussed in detail in Part II of this work; an enzyme, a phytosterol, fixed and volatile oil, and citrullol were also obtained.

According to the Kew Index<sup>(18)</sup>, there are two plants

which have received the name Leontice leontopetalum. One was named by Linnaeus and comes from Italy and the Middle East, and it is this plant which has been investigated in the present work. The other, named by Hooker, filius, and Thoms, was a misnomer, being synonymous with L. ewersmanni, and comes from Persia and Turkestan.

Leontice ewersmanni, Bge., was first investigated by Orekhov and Konovalova<sup>(29)(30)</sup> in 1932, who isolated a total of 0.4 per cent of alkaloids from the tubers of plant material collected in Central Asia. Analysis of the alkaloidal mixture showed the presence of three alkaloids, of which two, leontamine and leontidine, were characterised. Leontamine,  $C_{14}H_{26}N_2$  was obtained as a colourless oily base, b.p. 118-119°/4 mm.Hg., and was present to the extent of 0.075 per cent in the drug. Leontidine, a colourless crystalline alkaloid, m.p. 116-118° was obtained only with difficulty and in very small amounts. No empirical formula was assigned to this alkaloid. The third alkaloid was obtained only in the form of a crystalline picrate, m.p. 176-178°, from which the base could not be recovered. The presence of a fourth alkaloid in the plant may also be inferred from the authors' report that the basic (NaOH) liquid still gave alkaloid reactions after extraction with both ether and chloroform. The presence of a phenolic or quaternary-like alkaloid can therefore be inferred, on the

assumption that extraction with organic solvents was complete. Moreover the presence in the extract of a water-soluble alkaloid is possible since the primary extraction of the plant was with ammoniacal alcohol. However, apart from an attempted extraction of this base with phenol-chloroform (1-4), no further mention was made of this fraction. There was no indication of the content of this alkaloid in the plant. Yunusov and Sorokina<sup>(31)</sup> who reinvestigated the plant tubers in 1948, used dichloroethane as the extraction solvent and consequently did not report a water-soluble alkaloid; nor were the marcs left after extraction with dichloroethane tested for the presence of water-soluble alkaloids. These authors verified the presence, in the tubers, of leontamine and leontidine, and assigned to the latter the formula  $C_{15}H_{20}ON_2$ . They proposed a revised formula for leontamine  $C_{15}H_{26}N_2$ , assuming it to be an optical antipode of pachycarpine  $C_{15}H_{26}N_2$ , a base isolated from the upper portions of the plant but not from the tubers. However this hypothesis was not supported by analytical data for either leontamine or its salts.

The yield of total alkaloids (0.17%) from the plant tubers reported by Yunusov and Sorokina<sup>(31)</sup>, was very much less than that reported by Orekhov and Konovalova due to the fact that the tubers were collected when the fruit had begun to form. It is not surprising therefore that the reported yields of leontamine and leontidine were also

significantly different from those of the earlier workers. They also found that during the ripening period of the seeds, 0.44% of alkaloids were isolated from the leaves and stem, and 0.32% from the tubers, the latter being raised to 0.6% after the aerial portions of the plant had died down. Thus they assumed that Orekhov and Konovalova did their researches on tubers collected from plants during the period when the seeds were ripening.

Yunusov and Sorokina stated, in contradiction to Orekhov and Konovalova, that the alkaloid mixture contained about 50% of leontidine hydrochloride and only 5% of leontamine. They also isolated a third crystalline base leontine,  $C_{15}H_{24}ON_2$ , m.p.  $103-104^{\circ}$ , which, unlike leontidine was optically inactive. Two salts, a perchlorate and a methiodide were described, but the picrate was not prepared. It is uncertain therefore, whether or not this base is in fact identical with the third base isolated by Orekhov and Konovalova as the picrate. Leontine was present in the alkaloid mixture to the extent of 15% of the total base.

The same authors<sup>(31)</sup> have also investigated the aerial portions of L. ewersmanni, and found that, collected as before, they yielded 0.87% of a mixture of alkaloids from which leontidine (50% of total base, calculated as hydrochloride), pachycarpine (1.7%), d-lupanine (5%) and leontine (10%) were isolated. The alkaloid content of the

aerial portions of the plant was observed to be the greatest during the period of intensive growth, whilst that of the tubers was a minimum (0.17%) at this time. Little or no alkaloids remained in the dry stubble of the plant after the aerial portions had died away.

Platonova, Kuzovkov, and Massagetov<sup>(32)</sup> have recently isolated two new alkaloids, tapsine and isoleontine, from the upper portions of L. ewersmanni, again extracting with dichloroethane in the presence of ammonia. IsoLeontine was obtained as its picrate, m.p. 177-179° and may therefore be identical with the third base of Orekhov and Konovalova (picrate m.p. 176-178°). The free base, isoleontine, m.p. 107-108°,  $[\alpha]_D - 78.2^\circ$  (ethanol) was recovered from the picrate by successive treatment with hydrochloric acid and sodium hydroxide and formulated as  $C_{15}H_{24}ON_2$ . No chemical constituents, other than the above alkaloids have been reported for L. ewersmanni<sup>(33)(34)</sup>.

L. alberti, a small, uncommon, mountain plant from Central Asia, was also investigated chemically by Yunusov and Sorokina<sup>(31)</sup>, who found that the aerial portions collected at the beginning of the flowering period yielded 1.0% of total alkaloids. The latter consisted mainly of methylcytisine (37%) and very small amounts of a crystalline alkaloid, m.p. 180-183°, and a liquid base. The tubers, collected at the same time, yielded 0.75% of an alkaloid mixture containing



methylcytosine (60%) and a trace of the liquid base. The latter was not investigated further owing to the small quantity available. Again no other plant constituents were isolated.

PHARMACOLOGICAL ACTIONS OF THE BERBERIDACEAE ALKALOIDS  
AND OTHER CHEMICALLY RELATED ALKALOIDS

A few plants of the Leontice species have been investigated chemically<sup>(29)(30)(31)</sup>, but neither the plants nor the alkaloids isolated, have been tested for their pharmacological action.

Caulophyllum thalictroides, a species closely related to the Leontice species, has been chemically investigated<sup>(28)</sup> and the alkaloid and saponins isolated tested pharmacologically. The alkaloid methylcytisine, was found to be one tenth as potent as cytisine, which causes convulsions and death by respiratory failure. The saponins were non-toxic when given orally, a dose of 0.1g. in a small cat causing only a mild purgative action after several hours. Recently, however, Ferguson and Edwards<sup>(35)</sup> have claimed that caulosaponin has an oxytotic effect on isolated rat uterus.

Various other closely related species of the plant family Berberidaceae, have been investigated and the alkaloids present isolated as pure compounds. The principal alkaloid found in the Berberidaceae is berberine, which however has never been used or recommended for epilepsy. It has been shown to cause dyspnoea, and lower the blood pressure in experimental animals, but its main use is as a bitter tonic or stomachic on account of its intensely bitter

taste. Canadine [(-) tetrahydroberberine] in large doses causes transient excitement followed by depression and paralysis of the central nervous system. Its injection is followed by violent peristalsis and diarrhoea. Laidlaw<sup>(36)</sup> who studied the action of canadine- $\alpha$ -, and  $\beta$ -methochlorides, found both to have the curare-like action common to ammonium bases. Reynolds and Waud<sup>(37)</sup> found that capaurine produced paralysis when injected into the lymph-sac of frogs, and convulsions when injected into mice or rabbits in doses of 100-200 mg./Kg. According to Biberfeld<sup>(38)</sup>, palmatine, columbamine and jatrorrhizine, all paralyse the central nervous system in frogs, palmatine also producing this effect in mammals and differing from the other two in stopping respiration, probably by paralysis of the respiratory centre. Oxycanthine, administered in doses of 0.1g. to 0.2g. to rabbits, produces quick and laboured respiration, muscular tremors, clonic convulsions and cessation of respiration before the heart stops<sup>(39)</sup>.

Other alkaloids, chemically closely related to berberine (but obtained from plant families other than the Berberidaceae), such as cryptopine<sup>(40)</sup>, protopine<sup>(41)</sup>, corycavine<sup>(42)</sup>, corycavamine<sup>(42)</sup>, corytuberine<sup>(42)</sup>, glaucine<sup>(43)</sup>, dicentrine<sup>(44)</sup>, domesticine<sup>(45)</sup>, pukateine<sup>(46)</sup> and boldine<sup>(47)</sup> have all been shown to exhibit convulsant activity in experimental animals and in a great many cases, death is due to respiratory failure, presumably by paralysis of the respiratory centre.

DISCUSSION  
OF  
EXPERIMENTAL  
WORK

PRELIMINARY EXAMINATION OF LEONTICE LEONTOPETALUM

Sublimation of the powdered drug in vacuo<sup>(48)</sup>, produced small amounts of a yellow oily material which was almost insoluble in cold and hot water. On shaking with the latter solvent, the substance was divided into fat-like globules which floated on the surface of the water. These globules produced a "grease-spot" on paper, showing that the substance was fat-like. The oil, faintly alkaline in reaction, was only partly soluble in dilute mineral acids and the filtrate gave only a faint Mayer's reaction, indicating the presence of a small amount of a volatile base. It is to be noted, however, that the above process does not indicate whether or not the sublimed substance was present as such in the original material<sup>(49)</sup>.

Small quantities of the powdered drug were extracted with such solvents as water, dilute acid, and ethanol, and the extracts so obtained examined qualitatively. The solution from a warm (50°) aqueous extract gave no colouration with ferric chloride or precipitate with lead acetate, indicating the absence of tannin and anthoxanthins. However, with basic lead acetate, a voluminous buff precipitate was obtained indicative of glycosides. Some confirmation of this was obtained by the fact that the solution failed to exhibit reducing properties with Benedict's or Fehling's reagents, but did so after acid hydrolysis, although this test

is not necessarily conclusive, owing to the possible presence of starch in the extract. The aqueous extract also gave precipitates (alkaloid) with both Mayer's and Dragendorff's reagents.

Extraction of the drug with warm ( $40^{\circ}$ ) aqueous sodium carbonate solution (1%) to dissolve acidic material, yielded an aqueous solution which gave a copious and persistent froth having a characteristic honeycomb structure. The froth was not destroyed on boiling, and no precipitate was formed, and this, together with a negative Millon's test, showed water-soluble protein to be absent. Frothing was therefore indicative of a saponin and this was confirmed by the fact that the aqueous solution readily haemolysed red blood corpuscles. Cyanogenetic substances could not be detected in the plant.

The hot aqueous extract, after filtration and cooling, became gelatinous due to the large amount of starch present, and also contained a reducing substance and an alkaloid. Nevertheless, concentration of this extract and precipitation with ethanol did give a crude saponin fraction A (9.3%). The product, however, was very impure, and melted with decomposition over a range of  $22^{\circ}$ . This method<sup>(50)</sup>, of precipitating the saponin from aqueous solution was preferred to such methods as precipitation with barium hydroxide<sup>(51)</sup> or lead subacetate<sup>(52)(53)</sup>, the latter reagent having in fact

been shown to give a voluminous precipitate with the saponin. However, according to Kofler<sup>(54)</sup> such treatment results in considerable loss of material and is therefore to be avoided.

The presence of an alkaloid in the drug was confirmed by extraction with warm 1% aqueous hydrochloric acid, when a positive reaction with Mayer's reagent was obtained. The solution also gave characteristic alkaloidal reactions with phosphotungstic acid solution, iodine-potassium iodide solution, Dragendorff's reagent, and solution of picric acid.

The presence of saponin and alkaloids in the plant was confirmed by a more thorough preliminary examination carried out by the method of Stas-Otto<sup>(55)</sup>. This method depended on the fact that both alkaloids and glycosides (saponins) are extracted by alcohol containing 1% tartaric acid, and although the saponins and alkaloid tartrates are both soluble in water, only the former are extracted from aqueous solution with ether. This method gave only a very small amount of an ether-soluble saponin which, however, gave a positive Molisch's test (for carbohydrates and glycosides), was readily soluble in water, and only reduced Fehling's and Benedict's reagents after acid hydrolysis. The aqueous extract, after extraction with ether, showed evidence of three alkaloid fractions. Two were partly extracted with ether from caustic soda and ammoniacal solutions respectively, but were more readily extracted with chloroform, and gave alkaloidal reactions with the usual

reagents. The other, which was not extracted with either ether or chloroform from alkaline solution, gave positive reactions with the usual alkaloidal reagents, was assumed to be quaternary in type, and was precipitated as the Mayer-alkaloid complex.



THE GENERAL METHOD OF EXTRACTION (56)

The method adopted was to continuously extract the powdered drug (200g.) with a series of hot solvents (Method A.). The extracts obtained were dried in vacuo to constant weight and the residues examined qualitatively to give the results shown in Table 1.

In order to determine whether or not heat had caused any decomposition of the substances isolated, the powdered drug (100g.) was extracted by percolation to exhaustion with the same series of solvents (Method B), to give the results also shown in Table 1.

At this stage, apart from any apparent hydrolysis of the saponin, it was impossible to determine whether or not any decomposition of the alkaloid had occurred as in both methods, the alkaloids were obtained as dark sticky solids from which no crystalline material could be isolated.

The general method was applied to find the amount and nature of the material isolated by the various solvents. No attempt was made, at this stage, to characterise the substances isolated.

Petrol extracted fat, wax, fixed and volatile oil, and a phytosterol, whilst both ether and chloroform extracted only very small amounts of saponin and alkaloid. Extraction with ethanol on the other hand removed most, but not all of

the saponin and alkaloid present as well as colouring matter and other extraneous matter.

The percentages given for the constituents of each extract are only approximate, with the exceptions of those in Method 1. This is due to the fact that the saponin fractions isolated at this stage were impure, and the percentage of alkaloid was obtained by difference on the assumption that the material remaining after the separation of saponin was alkaloid. These alkaloid fractions were later shown to contain a good deal of colouring matter and appreciable amounts of saponin.

Solvent	Method	Weight extracted (g.)	% of the air-dried drug.	Nature	Constituents.				Comments
					Present	% of the hot extract	% of the drug	Absent *	
Light Petroleum	Hot (A)	0.444	0.22	Yellowish-brown semi-solid	Phytosterol Wax. Fat. Fixed oil Volatile oil	20 10 70 Trace	0.044 0.022 0.155 0.001	Saponin Alkaloids	+
	Cold (B)(1.5l.)	0.198	0.198						-
	(A)	0.875	0.44		Saponin Alkaloid Colouring matter.	56	0.245	Fat, etc.	-
Ether	(B)(2l.)	0.415	0.415	"		44	0.192	Reducing substances	-
	(A)	0.568	0.284		Saponin. Alkaloid. Colouring matter.	75	0.213	Reducing substances	-
Chloroform	(B)(0.9l.)	0.242	0.24	"		25	0.07		-
	(A)	44.57	22.28		Saponin Alkaloid. Colouring matter.	71	15.89	Reducing Substances	-
Ethanol	(B)(2.5l.)	18.03	18.03	"		29	6.41	Tannin	-
	(A)	2.86	1.43	Brown gummy solid	Saponin Alkaloid. Colouring matter. In the hot extract there was a trace of reducing substances.	60 40	0.85 0.57	Tannin	Cold - Hot +
70% ethanol	(B)(2l.)	0.978	0.98						
	(A)	0.98	0.49	Dark brown sticky residue	Saponin. Alkaloid Colouring matter. Reducing Substances.	75 25 Trace	0.384 0.12	Protein. Tannin	+
Cold water.	(B)(2l.)	0.54	0.54						
	(A)	15.87	7.93	Yellow amorphous solid	Starch Alkaloid. Saponin. Colouring matter. Reducing substances	100 Trace ?	7.93 - -	Protein	+
Boiling water.	(B)(1l.)	8.16	8.16						
	(A)	0.68	0.34	Brown sticky residue	Alkaloids. Saponin. Colouring matter. Reducing substances.	Trace 100	- ?	-	+
Cold 1% hydrochloric	(B)(1l.)	0.29	0.29						
	(A)	0.29	0.29						

Table 1. Yields and constituents of extracts of the drug.

\*  
+  
†

Substances for which specific tests were applied, but found to be absent.  
+ indicates that an apparent decomposition had occurred in the extract.  
Figures in parenthesis indicate the volume of solvent used.

ISOLATION OF AN ENZYME

It was shown in the preliminary examination (page 15) that free reducing substances (e.g. sugars) were not present in the plant. However, maceration, followed by percolation with water, slowly released reducing substances, and this was attributed to the presence of an enzyme. Accordingly the drug was extracted for the constituent enzyme by a standard method<sup>(28)</sup>. The grey amorphous solid obtained was dissolved in water, dialysed until free of reducing substances, and the solution tested for enzyme activity against standard solutions of a  $\beta$ -glucoside (Salicin) and an  $\alpha$ -glucoside ( $\alpha$ -methyl D-glucoside) and its action on these substances compared with a  $\beta$ -glucosidase. It was found (Table 2) that the solution caused slow hydrolysis of the  $\beta$ -glucoside only, the hydrolysing power being lost after boiling the solution for 10 minutes, thus showing that an enzyme (a  $\beta$ -glucosidase) was in fact present in the material isolated. Comparison of the enzyme with a known  $\beta$ -glucosidase has shown, (Table 2), that it is not very active, although it is possible that a good deal of the activity may have been destroyed by the method of isolation employed.

#### IV MAJOR STRUCTURES

The basic structure of the area was produced by the Caledonoid recumbent folding, which caused repetition of several formations from north-west to south-east. The key formation in the structure, the Perthshire Quartzite Series, has been particularly affected by repetition, so that it outcrops in three belts, the Ben-y-Gloe, Tummel and Cairnwell Belts.

The Tummel Belt, which in north-eastern Perthshire consists entirely of Killiecrankie Schist, is the lowest structurally, although it outcrops between the other two. It rests on the Blair Atholl Series to the north-east and forms part of the lower limb of the Ben Lui recumbent syncline. The Killiecrankie Schist is bounded on both sides by slides which cut out the flanking quartzite members of the Series.

The Tummel Belt passes to the Ben-y-Gloe Belt farther north-west by an isoclinal anticline of which the Blair Atholl Series forms the core. Across the nose of the anticline the Perthshire Quartzite Series changes in facies so that the Killiecrankie Schist is replaced by the Ben-y-Gloe Quartzite. The change, however, cannot be traced since the nose of the anticline has been eroded. The Ben-y-Gloe Belt itself forms an isoclinal synform with an envelope of Blair Atholl Series; its axial plane dips to the south-east.

From the Ben-y-Gloe Belt the Perthshire Quartzite Series passes round the nose of the Ben Lui recumbent fold to reappear in the Cairnwell Belt which lies on the upper limb of the Ben Lui Syncline. In doing so

Glycoside	Enzyme	Time in minutes and results of hydrolysis. + faint, ++ heavy, precipitate of Cu <sub>2</sub> O																					Hours
		10	20	30	40	50	60	70	80	90	100	110	120	150	180	210	240	270	300	330	360	←-14 hours -→	25
Salicin	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	++	++	++	++	++
	β-glucosidase	-	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-methyl D-glucoside	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	β-glucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	Boiled Enzyme	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Action of enzyme, and  $\beta$ -glucosidase on  $\alpha$  and  $\beta$ -glucosides; temperature  $40^\circ$ .

## ISOLATION AND CHARACTERISATION OF LEONTOSAPONIN

### Isolation of leontosaponin.

Rosenthaler<sup>(57)</sup> states that saponins are readily hydrolysed by enzymes, and even by prolonged boiling in water. Various extraction procedures were therefore examined in order to obtain the best yields of unhydrolysed saponin. The criterion for the latter was the absence of reducing substances at all stages during the isolation of saponin, preliminary tests having indicated that no free reducing substances were present in the plant. These tests had also shown that water could not be used to extract the saponin, as cold water caused slow enzymatic hydrolysis to occur, and boiling water caused a good deal of hydrolysis of the saponin. Water, also, did not completely extract all the saponin from the drug, and although the general method (page 19) suggested that hot ethanol extracted most, but not all, of the saponin, it was found that extraction with boiling ethanol (Method 1) for a period of 8 hours, gave complete extraction of the saponin (17.1 to 17.36% of crude saponin), without hydrolysis, the boiling ethanol causing rapid destruction of the enzyme present in the drug<sup>(58)</sup>. Three crude saponin fractions, B, C and D (Table 3) were obtained by fractional precipitation with ether. The success of this method, however, depended on the use of absolutely dry solvents and the maintenance of absolutely dry conditions,

otherwise gummy saponin products were obtained which were only with great difficulty filtered off and dried. The difficulty of maintaining absolutely anhydrous conditions on the large scale led to the abandonment of this method for the large scale isolation of leontosaponin.

Attention was now turned to the method used by Power and Salway<sup>(28)</sup> (Method 2) for the isolation of the saponin from Caulophyllum thalictroides. The drug was extracted with boiling ethanol (95%), concentrated to a soft extract, a little water added, and the solution steam distilled (3 hours) to remove the volatile oil. The non-distilled solution was extracted with hot amyl alcohol to give a crude saponin fraction, E (8.25%). It was observed, however, that steam distillation had caused some hydrolysis of the saponin and a modification of the above method was then examined.

Boiling absolute ethanol was used (Method 3), instead of 95% ethanol as extraction solvent, since the large amount of crude saponin deposited on cooling the extract, was then readily filtered and dried. The saponin was deposited as a yellow amorphous solid, fraction F. The filtrate did not reduce Fehling's solution, but again steam distillation (to remove volatile oil) for as short a period as 30 minutes, showed that some hydrolysis of the saponin was taking place. Much of the remaining saponin was removed by filtration at



this stage, as a dark yellow solid, fraction G. Residual saponin was isolated by extraction with hot amyl alcohol as a dark yellowish-brown solid, fraction H. A total yield of 16.34% of crude saponin was obtained by this method.

Since it was evident that steam distillation, even for short periods caused hydrolysis of the saponin, it was decided to remove the fatty material with light petroleum prior to extraction of the saponin with alcohol. The method (Method 4) finally adopted for the large scale extraction of the saponin was as follows:-

The drug was defatted by maceration, followed by percolation, with light petroleum and the brown, fat-like residue reserved for future examination. The dried drug was then extracted in a large Soxhlet with Industrial Methylated Spirit for 48 hours. The alcohol was concentrated to give a dark brown soft extract (5.4Kg.). 1.9Kg. of this extract was then redissolved in the minimum amount of boiling absolute ethanol, which on cooling deposited a large amount of a yellow amorphous saponin fraction, I (8.4%) which was washed with a small quantity of ethanol to remove adsorbed alkaloid. The filtrate and washings were again concentrated to a soft extract which was freed from saponin by treatment with a small amount of water, filtration of the precipitated saponin fraction J (2.4%), and extraction of the filtrate with hot (60°) amyl alcohol to give crude saponin, fraction K

(6.56%). The filtrate, after extraction of the saponin, was reserved for future examination of the alkaloids. Reducing substances were absent at all stages during the isolation of the saponin. This method gave the best yield (17.36%) of crude saponin.

The various methods used, and the saponin fractions obtained are shown in Table 3. It will be seen that the melting ranges suggest that an apparent separation into two saponin fractions had been effected, although both were obviously impure as shown by the colour and melting range. The various saponin fractions were therefore kept separate in the subsequent purification processes in order to ascertain whether in fact two or more saponins were present in the drug.

Method	Crude saponin fraction	Colour	m.p. (decomp.)	Yield (% of Hydrolysis drug)	
(page 16)	A	Yellowish-brown	190-212°	9.3	+
1.	B	Dark yellow	218-226°	11.4	-
	C	" "	224-231°	2.1	-
	D	Yellowish-brown	188-194°	3.6	-
2.	E	Dark yellow- ish brown	195-218°	8.25	+
3.	F	Yellow	217-221°	7.5	-
	G	Dark yellow	212-222°	2.14	+
	H	Dark yellow- ish brown	188-220°	6.7	+
4.	I	Yellow	219-236°	8.4	-
	J	Dark yellow	212-238°	2.4	-
	K	Dark yellow- ish brown	192-206°	6.56	-

Table 3. Comparison of the crude saponin fractions obtained.

### Purification of the saponin fractions.

Various methods were tried in order to obtain a colourless crystalline saponin. About 75% of each of the saponin fractions readily dissolved in hot ethanol. The remainder resisted solution, but was eventually soluble in a large volume of hot ethanol. It was thought at first, that a separation of saponins had been effected by this difference in solubility, but after decolourisation with charcoal and precipitation with dry ether, both fractions designated x and y respectively, in every case melted in the same range  $224-236^{\circ}$ , and all had specific rotations between  $+11^{\circ}$  and  $+14.5^{\circ}$ . Thus the apparent separation was due to the limited solubility of the saponin in ethanol. The saponin fractions were more readily soluble in hot methanol, but the products after treatment with charcoal were still pale yellow. Moreover, much of the saponin was adsorbed by the charcoal. Repeated precipitation from absolute methanol with dry ether also failed to remove the colour from the fractions. This method, however, was successful in removing the fatty material, which was shown by acid hydrolysis of one of the crude saponin fractions to be present in saponins isolated by Methods 1 to 3 inclusive.

Removal of the fatty material, however, did not result in a crystalline saponin and all attempts to crystallise the saponin fractions from ethanol or methanol failed, amorphous

products being obtained. It was apparent that either the saponin fractions were amorphous, or that some impurity was preventing crystallisation. It was found at this time, that appreciable amounts of inorganic salts were present. These were removed by dialysis<sup>(59)(60)(61)</sup>, this raising the melting point of all fractions to between 232 and 236° (decomp.), indicating that they might well be homogeneous and identical. The colouring matter in the various fractions was not removed by this process and the fractions varied in colour from buff to very pale yellow.

A further attempt was made to decolourise the saponin by the method of Rosenthaler<sup>(62)</sup>. This is based on the fact that whilst saponins and colouring matters form compounds with magnesia, only the saponin compound is decomposed by boiling ethanol with solution of the saponin in this solvent. However, application of this method to a portion of fraction G, gave a product which was still pale buff in colour and non-crystalline. The yield of recovered saponin was poor and since it was felt that even this mild alkali treatment was undesirable, the method was abandoned. A colourless crystalline saponin, m.p. 236-238° (decomp.) was finally obtained from one of the fractions by conversion to the corresponding acetate and hydrolysis of the acetate with barium hydroxide<sup>(63)</sup>. It was afterwards found that the pure saponin could also be obtained directly by trituration

of the crude fractions with the minimum amount of cold absolute ethanol which removed the yellow colouring matter. Repetition of this process gave material which was almost colourless, and which could be recrystallised from absolute ethanol. All the fractions gave the same crystalline saponin, m.p. 236-238° (decomp.). In order to verify that the cold alcohol had not removed a more readily soluble saponin, the combined alcohol filtrates were evaporated and the residue again treated as above, when a small amount of the same saponin was isolated. This fact was later verified by paper chromatography of the saponins, only one distinct spot of identical  $R_F$  value being obtained. The saponin present in the root-tubers to the extent of 17.3% (crude), had not been previously isolated and was designated leontosaponin.

#### Properties of leontosaponin.

Leontosaponin was obtained as colourless, micro-crystalline prisms, m.p. 236-238° (decomp.) with preliminary slight sintering at 231°,  $[\alpha]_D$ , + 15°. The saponin had an acrid taste and was very sternutatory. The pure saponin was moderately soluble in water to give a colourless colloidal solution which was slightly acidic in reaction and which when shaken gave a copious and persistent froth, having a marked honeycomb structure. An aqueous solution of the saponin

gave a voluminous white precipitate with a solution of basic lead acetate and also emulsified fixed and volatile oils, giving coarse, unstable emulsions. Aqueous solutions haemolysed red blood corpuscles in vitro, having a Haemolytic Index 1-100<sup>(64)</sup>, although in vivo the saponin was surprisingly non-toxic, having little observed lysogenic action.

Leontosaponin was soluble in large volumes of hot ethanol, but more readily so in hot methanol. It was almost insoluble in cold anhydrous ethanol, but more readily soluble in cold aqueous alcohols, and hot amyl alcohol. It was appreciably soluble in chloroform, but insoluble in dry ether, petroleum ether, benzene, carbon tetrachloride and acetone. It was also soluble in caustic alkalis indicating an acidic or phenolic group in the saponin.

The saponin did not reduce either Fehling's or Benedict's solution, but after acid hydrolysis, a gelatinous sapogenin was formed and the filtrate reduced both the above reagents, showing the presence of a reducing sugar. Leontosaponin gave positive reactions for colour tests characteristic of saponins. With concentrated sulphuric acid a play of colours was obtained, the material changing slowly (30 minutes) from bright red → blood red → violet → mauve → deep purple<sup>(65)</sup>. The process was accelerated by heat. A mixture of ethanol, and concentrated sulphuric acid containing a trace of ferric chloride, gave a bluish-

green colour with the saponin<sup>(66)</sup>. Acetylation of leontosaponin gave leontosaponin acetate as small colourless needles, m.p. 155-156°,  $[\alpha]_D +19.9^\circ$ . The acetate was insoluble in water, but readily soluble in ethanol, methanol, ether and chloroform. It was readily hydrolysed by alkalis to give the original saponin.



### ACID HYDROLYSIS OF LEONTOSAPONIN

The sugar chains of natural saponins are cleaved under acid conditions, and various strengths of acid have been used in the hydrolysis of different saponins. Wall, Rothman and Walens<sup>(67)</sup> studied the hydrolysis of a number of steroidal saponins and found that the saponins were rapidly hydrolysed (1-2 hours) with 4N hydrochloric acid, yielding in most cases 90% of the total sapogenin. Varying degrees of hydrolysis were obtained with 2N acid, even when the reaction time was extended from two to eight hours, but all experiments with N hydrochloric and N or 2N sulphuric acid were completely ineffective.

Hydrolysis of leontosaponin was therefore carried out in the first instance with 4N hydrochloric acid. It was found, however, that although hydrolysis was complete in 4 hours, considerable charring of the resulting sugars occurred, and the sapogenin produced was both impure and difficult to purify.

Power and Salway<sup>(28)</sup> found that hydrolysis of the saponins present in C. thalictroides, proceeded favourably with 2.66N hydrochloric acid in ethanol. Examination of this method showed that leontosaponin could be similarly hydrolysed in 6 to 8 hours with 2.66N hydrochloric or sulphuric acids in ethanol. A gelatinous product was

deposited in the early stages of hydrolysis which gradually became crystalline as hydrolysis proceeded. This product, the sapogenin, was separated as a pale brown crystalline solid which was washed free of acid and recrystallised to give a colourless crystalline product. The best yields of sapogenin (95-99%) were obtained by continuing hydrolysis for at least 8 hours, shorter periods resulting in incomplete breakdown of the saponin. This could be detected by paper chromatography of the sugar solutions obtained on hydrolysis, the saponin giving a distinctive  $R_F$  value, as described later.

A much cleaner product was obtained when methanol was used as the solvent instead of ethanol, the resulting sapogenin being pale brown, crystalline and easily purified; the sugar solution was also merely coloured yellow instead of brownish-yellow.

Pure leontosapogenin was obtained from the various products of acid hydrolysis described by recrystallisation from ethanol (80%) as colourless, well formed rhombic prisms, melting at  $332-333^{\circ}$ , with preliminary sintering at  $315-319^{\circ}$ ,  $[\alpha]_D^{25}$ ,  $+80^{\circ}$  in ethanol,  $+80.4^{\circ}$  in pyridine. It was insoluble in water, chloroform, ether, light petroleum, benzene, and carbon tetrachloride, sparingly soluble in ethanol, but more readily so in methanol and in diluted ethanols (60-95%); and was readily soluble in pyridine and in a pyridine-water mixture (1:1). Leontosapogenin was

acidic in reaction (pH 5.2 in 95% ethanol). The sapogenin could be titrated with aqueous alkalis to give precipitates of the sodium or potassium salts, both of which were readily soluble in ethanolic alkali. With concentrated sulphuric acid, leontosapogenin gave the same colour reactions as did the saponin.

#### Characterisation of leontosapogenin as hederagenin.

Two groups of saponins are found in plants. The most common is the triterpenoid group, the other, the rarer group of steroidal saponins<sup>(68)</sup>. The classification follows from the fact that the genins obtained on hydrolysis of the saponins are either triterpenoids or steroids. Analysis of leontosapogenin showed it to have an empirical formula  $C_{30}H_{48}O_4$  which is typical of triterpenoids, the steroidal genins generally having a  $C_{(27)}$  formulation. Steroidal sapogenins are also soluble in such solvents as benzene, whereas triterpenoid genins are not. The infrared spectrum of leontosapogenin showed none of the four bands typical of normal and isosteroidal sapogenins<sup>(69)</sup>, a further indication of triterpenoid character.

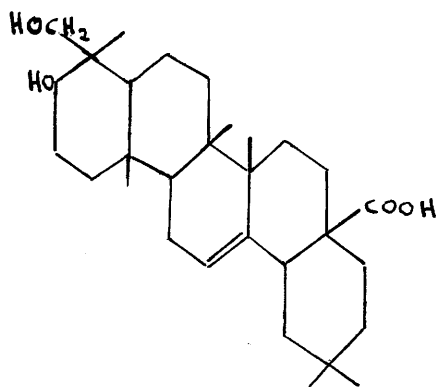
A literature search of known triterpenoid sapogenins suggested that leontosapogenin was probably identical with hederagenin, and this was confirmed by comparing leontosapogenin with an authentic sample of hederagenin from

soap-nuts, Sapindus saponaria<sup>(70)</sup>. They were found to have identical melting points<sup>(70)(71)</sup>, a mixed melting point showed no depression, and the rotations were identical in both cases<sup>(70)(72)(73)</sup>.

Hederagenin also occurs as the genin in a number of species, as  $\alpha$ -Hederin, from ivy leaves (Hedera helix. Family Araliaceae)<sup>(74)</sup>, Kalosaponin and Kalotoxin from the bark of Kalopanax ricinifolius (Family Araliaceae)<sup>(75)(76)</sup>, Mukurosin from the shells of soap-nuts, the fruits of Sapindus saponaria<sup>(70)</sup>, Linn., and S. mukurossi utilis<sup>(77)</sup>, and Akebia, from the branches of Akebia quinota<sup>(78)</sup>. It is also a constituent of the saponins from Egyptian Nigella sativa<sup>(79)</sup> and Aralia japonica<sup>(80)</sup>. Caulosapogenin, obtained from the saponin from C. thalictroides (Family Berberidaceae), a species closely related to L. leontopetalum, has also been shown to be identical with hederagenin<sup>(81)</sup> (see Part II).

The identity of leontosapogenin with hederagenin was confirmed by a detailed examination of its properties and by the preparation of a number of derivatives. The presence of two hydroxyl groups in leontosapogenin was established by conversion into a diacetate and a dibenzoate, both identical (melting point, mixed melting point, rotation and analysis), with genuine hederagenin diacetate and dibenzoate. The proximity of the two hydroxyl groups was confirmed by the formation of an isopropylidene derivative from the

corresponding methyl ester, identical in all respects with isopropylidene hederagenin methyl ester. The sapogenin gave a yellow colour with tetranitromethane and showed an ultra-violet absorption maximum (and absorption) at  $210 \text{ m}\mu$  ( $\epsilon$ , 2,860) indicative of a trisubstituted double bond as in authentic hederagenin. This bond was resistant to hydrogenation. Evidence of the carboxyl group was obtained by direct titration against standard sodium hydroxide, and by the isolation of the sodium salt and the methyl ester, identical in every respect with hederagenin methyl ester.



### hederagenin

The acid mother liquors obtained after the isolation of hederagenin were carefully neutralised with caustic soda solution. They gave reactions for reducing sugars and were examined as described in the next section.

ISOLATION AND IDENTIFICATION OF THE SUGARS FORMED  
BY ACID HYDROLYSIS OF LEONTOSAPONIN

The neutral sugar solutions obtained from the various hydrolyses of leontosaponin were bulked and the solution decolourised with animal charcoal. The resulting colourless liquor gave a positive reaction with Molisch's reagent (carbohydrate) and reduced both Fehling's and Benedict's reagents indicating the presence of reducing sugars. It did not give Seliwanoff's reaction for fructose, but gave positive reactions, with both Bial's and the Aniline reagent, for a pentose sugar. The sugar solution when heated with phenylhydrazine hydrochloride and sodium acetate for 10 minutes on a boiling water-bath deposited glucosazone, which was identified by its crystalline form (clusters of long slender needles) and melting point ( $201-204^{\circ}$ ). After rapid filtration whilst still hot, the solution was heated for a further 10 minutes, when a second crystalline osazone was obtained, which from its crystalline form (tangled masses of threadlike crystals) and melting point was shown to be arabinosazone. Traces of a third osazone which from its appearance may have been cellobiosazone, were found along with arabinosazone.

Further confirmation of the presence of glucose in the sugar solution was obtained by its reaction with lead acetate to give a salmon-pink precipitate typical of glucose, and by

the formation of saccharic acid (and silver saccharate), when the solution was oxidised with concentrated nitric acid. The formation of saccharic acid is specific for glucose even in the presence of other sugars such as arabinose<sup>(82)</sup>.

Chromatography of the sugar solution was done on paper by an ascending method, Williams and Kirby<sup>(83)</sup> claiming that this method gave more consistent results than the method of downward development. The solvent system used was n-butanol-ethanol-water (4-1-5)<sup>(84)</sup> and chromatograms were run for periods varying between 11 and 14 hours, when the sugar mixture was resolved into two spots, one being small round and compact and having an average  $R_F$  0.433, and the other larger, irregular and tailing, of  $R_F$  0.195. The latter was thought to be an unresolved mixture of sugars. The sugars were detected by spraying the dry papers with a mixture of 9 volumes of 0.2% naphthoresorcinol in n-butanol and 1 volume of 89% phosphoric acid<sup>(84)</sup>, mixed immediately before use. The paper after spraying was dried at 105° for 5 minutes, the sugars appearing as deep blue spots on a light purple background, which however darkened rapidly with final disintegration of the paper.

In an attempt to resolve the mixture of sugars completely, chromatograms were run for 20-24 hours and a 3.0% solution of p-anisidine hydrochloride in n-butanol<sup>(85)</sup> used as the spray reagent. This reagent gives a cherry-red colour with

pentoses, a green to brown colour with aldohexoses and a yellow colour with ketohexoses. The papers after spraying were dried at  $100^{\circ}$  for exactly 10 minutes, otherwise the specific colours were lost, the whole paper becoming brown in colour. Three distinct spots were obtained under these conditions. One, a small faint yellow compact spot, had an  $R_F$  averaging 0.432. The other two spots were larger and tailed slightly, one being cherry-red in colour (pentose)  $R_F$  0.260, and the other yellowish-brown in colour (hexose)  $R_F$  0.217. The spot of  $R_F$  0.432 did not correspond with the  $R_F$ 's of any known sugars, a number of which were run on paper as controls. It was thought that this spot might be due to traces of unhydrolysed saponin in the sugar solution, since some of the sugar solutions were from hydrolyses giving only 90-95% of the expected sapogenin yield. That this was the case was verified by direct comparison with leontosaponin which was shown to have an  $R_F$  0.433. The  $R_F$  values of the remaining two spots were identical with those found for genuine solutions of arabinose and glucose. Separation of the spots was, however, only slightly hindered by tailing, despite the large concentration of salt present in the solution, as was seen by chromatographing the salt-free sugar solution (prepared as below) on paper as before. (Table 12, page 120). Chromatography of the sugar solution on a column of paper-pulp and activated charcoal (1:1)<sup>(86)</sup> removed most, but not all, of the salts. All the sugars could be eluted



from the column with water, indicating that monosaccharides only were present (since disaccharides and trisaccharides require 5% and 15% aqueous ethanol respectively for elution).

Chemical separation of the sugars<sup>(87)</sup>.

Salt formation was later avoided by hydrolysing the saponin with dilute sulphuric, instead of hydrochloric, acid, when sulphate could be removed by neutralisation with barium carbonate. The salt free sugar solution was evaporated to dryness under reduced pressure. Part of the residue, in ethanol (50%) treated with N,N-benzyl phenylhydrazine in ethanol in a sealed vessel for 30 hours, gave arabinose N,N-benzyl phenylhydrazone, identical, melting point, mixed melting point and analysis, with genuine material. The mother liquors were refluxed with formaldehyde to regenerate the glucose, the oily formaldehyde osazone removed and the sugar solution evaporated to give a residue which did not contain any pentose sugar. This residue was treated with an ethanolic solution of N,N-diphenylhydrazine to give glucose N,N-diphenylhydrazone identical with authentic material. The sugars were regenerated from their respective osazones by refluxing with formaldehyde as before. The pentose sugar was recrystallised from boiling ethanol to give a white crystalline solid, whose melting point, rotation and colour reactions showed it to be L-arabinose. The hexose sugar was refluxed with acetic anhydride and sodium acetate to

give D-glucose  $\beta$ -pentaacetate, identical, (melting point, mixed melting point and rotation) with authentic D-glucose  $\beta$ -pentaacetate.

# CONSTITUTION OF LEONTOSAPONIN

From the analytical data on both leontosaponin and its acetate, and from the percentage of genin obtained on acid hydrolysis of the saponin, it appeared that the saponin molecule included a sugar chain of 6 or 7 monosaccharide units. The ratio of glucose to arabinose was determined by the method of Hirst and Jones<sup>(88)</sup>. This method depends on a paper chromatographic separation of the sugars and isolation of the individual sugars by elution with water. The amounts of sugars used in a series of experiments varied from 0.42mg. to 0.77mg. Periodate oxidation experiments on the isolated sugars indicated that glucose and arabinose were present in the proportions of 4:3 respectively. This result, together with the percentage yield of sapogenin (29.2 to 29.3%) from leontosaponin, indicated the empirical formula of leontosaponin to be  $C_{69}H_{112}O_{36}$ . This formulation, however, would require analyses to give C, 54.66; H, 7.43 per cent, whereas there was found for leontosaponin C, 51.5; H, 8.0 per cent. This discrepancy was readily accounted for by the fact that the saponin was found to contain 5 molecules of water of crystallisation, lost only on drying the saponin to constant weight at  $120^{\circ}$ . The empirical formula  $C_{69}H_{112}O_{36} \cdot 5H_2O$  is not only in complete agreement with the microanalytical data and yield of sapogenin, but also leads to an acetate which would analyse as found. No matter how the sugar chains are

arranged in the molecule, there must be twenty acetyltable hydroxyl groups. Complete acetylation therefore gives an empirical formula  $C_{109}H_{152}O_{56}$  for the acetate which requires C, 55.5; H, 6.5 per cent, in agreement with the analytical data found for the acetate, C, 55.7; H, 6.7 per cent.

#### Enzymatic hydrolysis of leontosaponin.

Hydrolysis of an aqueous solution of leontosaponin (pH 6.6) with a standard solution of  $\beta$ -glucosidase showed the saponin to be a  $\beta$ -glucoside, slow hydrolysis occurring after three hours at  $40^{\circ}$ . The saponin solution was also hydrolysed, after about 5 hours at  $40^{\circ}$ , by a standard solution of the enzyme isolated from the root-tubers of L. leontopetalum.

The hydrolysis rate was greatly increased at pH 4.4 at  $40^{\circ}$ (89), but since the control was also slowly affected this was probably a function of the acidity of the solution. The results of both of these experiments are shown in Tables 4 and 5 respectively.

Glycoside	Enzyme	Time in minutes and results of hydrolysis. + faint, ++ heavy, precipitate of Cu <sub>2</sub> O																						Hours
		10	20	30	40	50	60	70	80	90	100	110	120	150	180	210	240	270	300	330	360	←-14 hours -→	25	
Leontosaponin	β-glucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	++	++	++	++	++	
	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	++	++	
	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	

Table 4. Enzymatic hydrolysis of leontosaponin at pH 6.6

Glycoside	Enzyme	Time in minutes and results of hydrolysis. + faint, ++ heavy, precipitate of $\text{Cu}_2\text{O}$																					Hours
		10	20	30	40	50	60	70	80	90	100	110	120	150	180	210	240	270	300	330	360	←-12 hours -→	
Leontosaponin	$\beta$ -glucosidase	-	-	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	Enzyme	-	-	-	-	-	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	++	++	++	++

Table 5. Enzymatic hydrolysis of leontosaponin at pH 4.4

ISOLATION AND CHARACTERISATION OF LEONTICINE,  
LEONTAMINE, AND PETALINE

Preliminary investigations had shown the drug to contain three alkaloids. Two were soluble in chloroform, being extracted from caustic soda solution and ammoniacal solution respectively, the third a water soluble, quaternary-like alkaloid was not extracted from ammoniacal solution with either ether or chloroform. Extraction of the alkaloids was considerably handicapped by the presence in the plant of the large amount of saponin, and the alkaloidal fractions obtained were contaminated with saponin and difficult to purify. As with the saponin, where a method was sought which would give the best yields of unhydrolysed saponin, so various methods of extracting the drug were examined in order to find the best method of isolating the alkaloids. A method was also sought from the first, which could be used in the large scale extraction of both alkaloids and saponin, as the amount of drug available was limited.

According to Rosenthaler<sup>(90)</sup> there are three general methods of extracting alkaloids from plants.

- I Extraction with neutral solvents, such as ethanol and water.
- II Extraction with weak acids e.g. 1 per cent hydrochloric, or tartaric acids.

III Preliminary treatment of the drug with a base such as calcium hydroxide, or ammonia, in order to liberate the alkaloids from their salts, and extraction of the latter with an organic solvent (other than ethanol).

I Water is not a good solvent for alkaloids, even if the latter are present as salts, as is usually the case.

The alkaloid fractions obtained are usually very impure, particularly if hot water is used. Ethanol is a much better solvent for alkaloids, but extraction is not always complete, and again the extracts are usually very impure.

II Dilute acid usually extracts all the alkaloid, but again a good deal of impurities are also extracted. Heat should not be used, as in those cases where the alkaloids are present as esters, some hydrolysis of the ester may occur<sup>(91)</sup>.

III This method is usually the method of choice and generally yields fairly pure alkaloids. Water-soluble or quaternary alkaloids are, however, not extracted by this process, and again the use of heat may cause some hydrolysis of alkaloids which are present as esters<sup>(91)</sup>.

#### Extraction of the drug by method I

(a) Water. Preliminary tests (pages 15 and 16) had shown that both warm (50°), and boiling, water extracted some of

the alkaloids from the drug. Extraction of the marcs with 1% hydrochloric acid showed that much alkaloid remained unextracted by both warm and boiling water. The aqueous extracts were bulked, acidified, filtered, basified with ammonia, and extracted with chloroform to give a chloroform-soluble alkaloid, fraction A. A water-soluble but chloroform-insoluble, alkaloid, fraction B, was isolated by precipitation as the potassium mercuric iodide (Mayer) complex<sup>(92)</sup>.

(b) Ethanol. Cold percolation of the drug with ethanol extracted only a portion of the total alkaloid in the drug. Boiling ethanol, however, achieved a much higher extraction rate and efficiency. However, it was found in the early work that extraction of the alkaloids by this method was incomplete even after 8 hours extraction, and examination of alternative methods was therefore continued. Saponin was removed from the ethanol extracts, as already described, and the alkaloid residues separated as before into a chloroform-soluble fraction, C, and a water-soluble fraction, D, the latter being isolated from acid solution, as the Mayer complex. The alkaloid fractions so obtained still contained appreciable amounts of saponin and this seriously hampered purification.



Extraction of the drug by method II

Maceration, and subsequent percolation of the drug with 1% hydrochloric acid completely extracted all the alkaloids. A crude chloroform-soluble alkaloid fraction, E, and a water-soluble alkaloid fraction, F, (isolated as the Mayer complex) were isolated as before. Much extraneous matter was extracted by the large volumes of acid necessary for complete extraction of the alkaloids. Saponin also was extensively hydrolysed, and for these reasons the method was regarded as unsatisfactory. Precipitation of saponin and colouring matter from the acidic extract, by treatment with lead subacetate solution, caused much of the alkaloid to be lost by adsorption on to the precipitate, from which it was not readily recovered. Treatment of the acid solution with activated charcoal similarly removed the alkaloid, in addition to colouring matter and saponin; again recovery was difficult. Chloroform extraction of the solutions treated as above, and precipitation of the water-soluble alkaloid as before, gave the alkaloid fractions, G, and H, respectively, which were much cleaner than those previously obtained.

Both ether and chloroform also extracted non-alkaloidal impurities from the acid solution, but the process was extremely lengthy owing to persistent emulsion formation, and had to be abandoned.

The crude alkaloid fractions obtained in methods I and II.

(a) The chloroform-soluble alkaloids. The chloroform-soluble alkaloid fractions A, C and E were bulked to give a dark brown, gummy residue which could not be dried to a powderable solid in vacuo. Neither could it be induced to crystallise from any of the usual solvents, or mixture of solvents. The crude alkaloid residue was only partly soluble in dilute acid, due to the presence of saponin, which was thus removed. The material, purified in this way, and re-extracted from alkaline solution with chloroform, was, however, still obtained as a dark brown, sticky non-powderable residue. It gave a gummy picrate and hydrochloride, both of which resisted all attempts at crystallisation and purification, indicating that this fraction probably consisted of a mixture of alkaloids.

The chloroform-soluble fraction could be resolved into what appeared to be non-phenolic and phenolic fractions, I and J respectively, by extracting with chloroform, from caustic soda, and ammoniacal solutions respectively. The phenolic properties of fraction J were ascribed to it solely on the basis of the extraction procedure which was used, but careful examination of this fraction showed it to be insoluble in caustic alkalis, and it did not give the usual reactions for phenols. Most of it was soluble in water, whilst the remainder could be re-extracted from caustic soda solution

with chloroform. Thus it would appear that this small "phenolic" fraction consisted mainly of water-soluble alkaloid, together with a trace of chloroform-soluble alkaloid, and this was later verified (page 169 ) by paper chromatography of the reineckate of this fraction.

The chloroform-soluble alkaloid fraction, I, could not be dried to a powderable solid in vacuo, indicating that an oily base might be present in this fraction, nor would it form a crystalline picrate or hydrochloride.

The partly purified chloroform-soluble alkaloid fraction, G, similarly could not be dried in vacuo, nor did it form a crystalline picrate or hydrochloride, again indicating a mixture of alkaloids in this fraction.

(b) Water-soluble alkaloid fractions. The Mayer-complexes, fractions B, D and F were bulked and various methods of regenerating the water-soluble alkaloid from the complex examined. Treatment with both hot and cold aqueous sodium carbonate solutions<sup>(93)</sup> decomposed most of the complex, but the best recovery of alkaloid was obtained by using a warm (40°) solution of caustic soda<sup>(93)</sup>. The product, a dark brown gummy solid, which was obviously impure, could not be reduced to a dry powder, or crystallised from any of the usual solvents, and failed to yield a crystalline picrate or hydrochloride. It was only very slightly soluble in

ether and chloroform, but very soluble in water and alcohol.

The partly purified complex, fraction H, similarly gave a dark-brown sticky residue which could not be crystallised, and failed to form crystalline derivatives.

Extraction of the drug by method III. The alkaloids were extracted with benzene after treating the drug with ammonia. The benzene-soluble extract, a dark-brown sticky alkaloidal extract, of which only a trace was water-soluble, was free from saponin. Extraction of the marc with boiling ethanol extracted the water-soluble alkaloid, which was precipitated from acid solution as the phosphotungstic acid complex<sup>(94)(95)</sup>, fraction K. This method suffers from the disadvantage that two extractions are necessary to extract the alkaloids and the use of alkali was shown to cause some hydrolysis of the saponin to occur. The phosphotungstic acid complex too, was obtained as a slimy solid, which was only filtered and dried with great difficulty.

A modification of this method to overcome the necessity of a dual extraction was adopted, the alkaloids being liberated with ammonia or calcium hydroxide, and extracted with ethanol.

Treatment with calcium hydroxide and percolation with 70% ethanol gave an extract which contained some but not all of the alkaloid present. A further disadvantage was the

very large volume of solvent essential to the method. More efficient extraction was obtained with 90% ethanol, whilst the highest yields of alkaloid were obtained with absolute ethanol. After precipitation of saponin from the bulked extracts, chloroform-soluble alkaloids were extracted from ammoniacal solution to give fraction L. The remaining water-soluble alkaloid was precipitated from acid solution with mercuric chloride solution<sup>(96)</sup>, when an easily filterable complex, fraction M, was obtained. The yields of alkaloids were not increased by moistening the drug with ammonia (10%) instead of calcium hydroxide, prior to percolation with absolute ethanol.

The crude alkaloid fractions obtained by method III.

(a) The chloroform-soluble alkaloid still contained an appreciable amount of saponin. Ion exchange resins (Zeo-Karb 225<sup>(97)</sup> and Amberlite I.R.C. 50<sup>(98)(99)</sup>) removed the saponin from the extract, but did not remove colouring matter. Their main disadvantage was the fact that the alkaloid was strongly adsorbed, and not easily, nor fully, recovered. There was no apparent separation of bases on the resin columns. Saponin was best removed from the crude extract by continuous extraction of the extract with dry benzene in a Soxhlet apparatus. The alkaloid together with some colouring matter was soluble in the benzene, but saponin,

and a small portion of alkaloid (found to be soluble in water), remained undissolved.

A portion of the benzene-soluble alkaloid was used to prepare a reineckate<sup>(100)(101)</sup> which was purified by recrystallisation from aqueous acetone, to yield a pale pink amorphous solid m.p. 180-182° (decomp.). Chromatography of this reineckate on paper<sup>(102)</sup>, using upward development for 16 hours with the solvent system, pyridine-water (1-4)<sup>(102)</sup>, and spraying with a solution of potassium bismuth iodide, showed the presence of three alkaloids in this fraction. Spots of  $R_F$  values 0.92, 0.62, 0.5 were obtained, the first two spots being compact. The first one particularly was very faint and was a brownish colour, in contrast to the pale pink colour of the other two, and may possibly be due to a trace of impurity in the reineckate. Most of the reineckate appeared to be that of  $R_F$  0.5.

Chromatography of the benzene solution of the alkaloid on a column of alumina<sup>(103)</sup> caused a partial separation of the alkaloids into two fractions. One could be completely eluted with benzene to give 76.5% recovery of base, the other was eluted only with benzene containing 1% ethanol to give 22.7% recovery of base. A small amount of each fraction was used to prepare reineckates, designated (i) and (ii) respectively, which were this time purified by chromatography on a column of alumina<sup>(104)</sup> to give in each case a crystalline

reineckate melting with decomposition at  $219-221^{\circ}$ . Paper chromatography of these reineckates<sup>(102)</sup>, however, showed that both were mixtures of two reineckates,  $R_F$  0.72 and 0.44 respectively. In reineckate (ii) the amount of reineckate of  $R_F$  0.72 was apparently much smaller than in reineckate (i) but in both cases the bulk of the fractions appeared to be that of  $R_F$  0.44. Thus no useful separation of the alkaloids had occurred on the column.

The benzene solutions were bulked and evaporated to give a dark-brown, sticky semi-solid, which again could not be dried to a powderable solid in vacuo. It was dissolved in dilute acid, basified with ammonia, and separated into an ether-soluble, and a chloroform-soluble alkaloid, by shaking with ether, and then chloroform. Again reineckates were prepared, purified, and chromatographed on paper as before<sup>(96)</sup>, and showed that both fractions were mixtures of two alkaloids of  $R_F$  0.72 and 0.44. The reineckate from the ether-soluble fraction, however, contained much less of fraction of  $R_F$  0.72, than that from the chloroform-soluble fraction.

The ether-soluble fraction was obtained as a pale-brown oil, from which a very small amount of a colourless alkaloid, m.p.  $118.5-119.5^{\circ}$ , separated. The chloroform-soluble alkaloid fraction could not be induced to crystallise, nor would it form a crystalline picrate, oxalate or hydrochloride. Thus the bulk of both fractions could not be crystallised.

A small scale distillation at this stage produced a yellow oily alkaloid, b.p.  $150-160^{\circ}$  (bath)/4 mm.Hg., and indicated that the majority of both these fractions may be an oily alkaloid. Lack of material prevented further confirmation at this stage.

(b) Water-soluble alkaloid complexes.

(i) The phosphotungstic acid complex, fraction K, was decomposed with hot barium hydroxide solution<sup>(94)</sup> to give a dark-brown gummy alkaloid, which could not be dried in vacuo nor crystallised from the usual solvents. It failed to give a crystalline hydrochloride or picrate, and was thus either a mixture of alkaloids, or very impure.

(ii) The mercuric chloride complex, fraction M, was decomposed in ethanol with hydrogen sulphide<sup>(96)</sup> to give the quaternary chloride as a dark-brown, powderable solid, which was deliquescent and could not be crystallised. It could not be completely decolourised with charcoal, and was obtained as a yellow, scale-like, powderable solid, melting with decomposition between  $130^{\circ}$  and  $140^{\circ}$ , the melting point being difficult to determine on account of the ease and rapidity with which the solid absorbed moisture. The chloride so purified, could not be crystallised from the usual solvents.

All the precipitating agents which had been used so far,



suffered from the disadvantage that impurities were precipitated along with the alkaloid. In every case it was impossible to purify the precipitated alkaloid complex, so that regeneration of the alkaloids had always given a very impure water-soluble alkaloid. It was found, in the purification of the chloroform-soluble alkaloids, that precipitation of the alkaloid as the reineckate gave a complex which could be purified by chromatography on a column of alumina. Decomposition of the pure reineckate gave pure alkaloids, and this method was subsequently adopted as the method of choice in precipitating the water-soluble alkaloid.

Summary of the methods examined. Of the three general methods, Methods II and III (using ethanol as solvent) were very successful in isolating the alkaloids, although in both methods the alkaloids obtained were very impure. However, these methods caused hydrolysis of the saponin, and as a method was being sought which could be used to extract both saponin and alkaloids on the large scale, these methods were abandoned. The use of water, in Method I, was also rejected on account of the poor solvent properties for both alkaloids and saponin. Hot ethanol, on the other hand was the solvent of choice in the extraction of saponin. It was also a good solvent for the alkaloids, extracting most, though apparently not all, the alkaloids. However, according to Hamerslag<sup>(105)</sup> hot ethanol will extract all the alkaloid from plants without

preliminary treatment of the drug with alkali. The use of hot ethanol on a small scale showed that prolonged extraction with hot ethanol in a Soxhlet (48 hours) did eventually remove all the alkaloids present, without hydrolysis of the saponin and without previous basification of the drug. The method adopted for the large scale extraction of the drug Method 4 (page 25) has already been given for convenience in the work on the isolation of the saponin. This method used hot Industrial Methylated Spirit for the extraction of both saponin and alkaloids from the drug.

#### LARGE SCALE ISOLATION OF THE ALKALOIDS

- (a) The mother-liquors, obtained after extraction of the saponin from the 1.9Kg. of crude extract from L. leontopetalum, were basified with ammonia and extracted (to exhaustion) with chloroform, to give a dark-brown, crude alkaloid fraction. The water-soluble alkaloid was isolated from the ammoniacal filtrate by acidification and precipitation with ammonium reineckate solution<sup>(100)(101)</sup> to give a brownish red, impure reineckate, melting with decomposition at 160-180°.
- (b) The remaining 3.5Kg. of the crude extract obtained from the root-tubers of L. leontopetalum, was treated with dilute sulphuric acid, filtered from precipitated saponin, (which was rejected) the filtrate basified with ammonia and

extracted with chloroform to give a dark-brown, crude alkaloid fraction. The water-soluble alkaloid was isolated as the crude reineckate, as before.

#### Purification of the chloroform-soluble alkaloids.

The chloroform-soluble alkaloid fractions were bulked, absorbed on to cellulose powder, and extracted in a Soxhlet with dry benzene, to separate the alkaloid from an appreciable amount of non-alkaloidal material which contained saponin, and traces of a water-soluble alkaloid. The oily extract obtained was dissolved in dilute acid and repeatedly extracted with ether, and then chloroform, to remove non-alkaloid impurities<sup>(106)</sup> which were rejected. In neither case was this extraction hindered by emulsion formation, as all the saponin had been removed. The acid solution was now basified with ammonia and extracted first with ether, and then chloroform.

The ether extract. Evaporation of the washed, and dried, ether extracts, gave a pale-brown viscid oil, which finally yielded from methanol (80%), a small amount (equivalent to 0.007% of the root-tubers) of a colourless crystalline alkaloid, melting at 118.5-119.5°. The bulk of the oily, ether extract, failed to crystallise and was distilled under reduced pressure, when only one fraction was obtained as a colourless oily alkaloid, b.p. 118-120°/4 mm.Hg.

The chloroform extract. Evaporation of the dried chloroform extracts gave a brown viscid oil, which again partly crystallised, giving a further yield of the crystalline alkaloid melting at  $118.5-119.5^{\circ}$  (equivalent to 0.0114% of the root-tubers). Most of the chloroform-soluble fraction also failed to crystallise, and was distilled in vacuo, as before, to give the same colourless oily alkaloid, b.p.  $118-120^{\circ}/4$  mm.Hg.

THE CRYSTALLINE ALKALOID, LEONTICINE.

The alkaloid was present in the root-tubers to the extent of 0.018 per cent., and was obtained as small glistening needles, m.p. 118.5-119.5°. It was optically inactive and had an empirical formula  $C_{20}H_{25}O_3N$ , which was confirmed by preparation of a crystalline hydrochloride, picrate, and chloroplatinate. Thus it was not identical with leontidine<sup>(31)</sup>, from L. ewersmanni, which although it has the same melting point 118-119°, is strongly laevo-rotatory,  $[\alpha]_D -188.7^\circ$  and has an empirical formula  $C_{15}H_{20}ON_2$ . Neither can it be equated to leontine<sup>(31)</sup>, m.p. 103-104°, which is also obtained from L. ewersmanni, and is also a di-acid base of formula  $C_{15}H_{24}ON_2$ . The alkaloid obtained is mono-acidic, appears to be a new alkaloid and has been designated leonticine. Leonticine slowly decolourised acid potassium permanganate but could not be hydrogenated. The very low yield obtained has restricted further investigation of the alkaloid.

THE OILY ALKALOID, LEONTAMINE. This alkaloid is present in the root-tubers to the extent of 0.082 per cent., has a b.p. 118-120°/4 mm.Hg.,  $n_D^{20}$  1.5117,  $d_{20}^{20}$  0.987, and  $[\alpha]_D^{20} + 2.78^\circ$ , in ethanol. This suggested that the base was probably identical with leontamine, the oily alkaloid isolated by Orekhov and Konovalova<sup>(29)(30)</sup> from L. ewersmanni, which has a b.p. 118-119°/4 mm.Hg.,  $n_D$  1.5113,  $d_{20}^{20}$  0.9880, and  $[\alpha]_D^{15} + 2.53$ , in absence of solvent. Analysis of the base and preparation of a number of derivatives showed that this oily alkaloid was in fact identical with leontamine, confirming the empirical formula  $C_{14}H_{26}N_2$  proposed by Orekhov and Konovalova<sup>(29)(30)</sup>. The contradictory formula  $C_{15}H_{26}N_2$  suggested by Yunusov and Sorokina<sup>(31)</sup>, but unsupported by experimental evidence is therefore incorrect.

Leontamine could not be hydrogenated, did not decolourise permanganate in acid solution, did not give a yellow colour with tetranitromethane, and is therefore fully saturated. Examination of the ultra-violet absorption spectrum of leontamine showed no absorption, thus confirming that the base was fully saturated. Formulation of leontamine as a di-tertiary base  $C_{14}H_{26}N_2$  which is completely saturated, rules out the possibility of an open chain structure, and suggests a saturated, fused system of a diaza-anthracene or phenanthrene type.

Further work on the base was restricted, due to lack of material.

THE WATER-SOLUBLE ALKALOID, PETALINE.

The crude reineckate was dissolved in dry acetone, filtered from a large amount of a brown amorphous, non-alkaloidal solid. Chromatography of the solution on a column of alumina<sup>(104)</sup> and elution with acetone showed only one alkaloid fraction to be present. Concentration of the eluate, precipitation of the reineckate with water, and careful crystallisation of the product from aqueous acetone gave a pink, micro-crystalline, reineckate, m.p.179-181.5° (decomp.). Two yellow bands, one before, and one immediately following the reineckate band, which were also eluted from the column were non-alkaloidal in character. The pure reineckate was decomposed by treatment of an acetone solution of the reineckate successively with silver sulphate, and barium chloride<sup>(101)</sup>. The combined silver reineckate, and barium sulphate precipitates, were filtered, washed with aqueous acetone, and the filtrate and washings evaporated to dryness in vacuo, at a temperature not exceeding 50° to give a greenish-yellow, deliquescent, scale-like solid. Solution in a small volume of methanol, removed a small amount of barium chloride. Repeated solution of the solid in water, removed quite an appreciable amount of a green, amorphous, insoluble solid (chromic sulphate). Evaporation of the filtrate gave a deep yellow, scale-like solid, which was deliquescent, and could not be crystallised. It gave all the usual alkaloidal reactions, but did not contain saponin.

The yellow colour could not be removed by charcoaling aqueous or alcoholic solutions of the base chloride obtained. The base chloride was soluble in water, ethanol, methanol, acetone and acetic acid. It was insoluble in benzene, light petroleum, carbon tetrachloride and n-butanol, but was slightly soluble in ether and chloroform. All attempts to crystallise the alkaloid chloride, which behaved as a typical quaternary salt, failed, owing to the facility with which it absorbed moisture. On drying in vacuo, it melted with decomposition at  $140-143^{\circ}$  (in a sealed tube), and was optically active,  $[\alpha]_D +11.3^{\circ}$ . The solid fluoresced a bright yellow in ultraviolet light, but the aqueous solution gave only a faint, blue-green fluorescence. The base chloride gave colour reactions<sup>(107)</sup> typical of a berberine type of alkaloid. Analytical data showed the base chloride to have an empirical formula  $C_{20}H_{22}O_3NCl \cdot H_2O$ . This formula, and constants of the base chloride, did not correspond with those of known bases and it was therefore designated petaline chloride. The above formula was confirmed by the preparation of a number of derivatives, including a picrate  $C_{20}H_{22}O_3N \cdot OC_6H_2O_6N_3$ , a chloroplatinate  $[C_{20}H_{22}O_3NCl]_2 PtCl_4$ , and the reineckate  $C_{20}H_{22}O_3N \cdot [Cr(SCN)_4(NH_3)_2]$ . A few other salts, such as the sulphate, nitrate, iodide and perchlorate were prepared, but were hygroscopic and could not be used for characterisation purposes. Petaline chloride gave a negative Labat test<sup>(108)</sup>, and a methoxyl determination<sup>(109)</sup> showed the



presence of two methoxyl groups in the molecule. The third oxygen was not present as an alcoholic, phenolic, aldehydic or ketonic group. It was thought, therefore, that it might be present as a third methoxyl group, as according to Pailer,<sup>(110)</sup> Belohlav and Simonitsch, methoxyl groups in some aromatic molecules require prolonged boiling with hydriodic acid. This method, heating for 6 hours, showed evidence of 2.5 methoxyl groups, and increasing the time to 8 hours, did not increase this figure. Thus a third methoxyl group could not with certainty be said to be present in petaline, and the fractional methoxyl group may have been due to interference by groups other than methoxyl e.g. a difficultly cleaved  $\text{N-CH}_3$  group. Thus the true nature of the third oxygen in petaline has not been determined.

#### Dihydropetaline chloride.

Reduction of petaline chloride with hydrogen in the presence of a platinum catalyst<sup>(111)</sup> gave dihydropetaline chloride as a colourless, hygroscopic solid, m.p.  $122-125^\circ$  (decomp.),  $[\alpha]_D -16.7^\circ$ , which was formulated  $\text{C}_{20}\text{H}_{24}\text{O}_3\text{NCl} \cdot 2\text{H}_2\text{O}$ . This formulation was verified by the preparation of a crystalline picrate, m.p.  $161.5-162.5^\circ$  (decomp.) and a crystalline chloroplatinate, m.p.  $179-180^\circ$  (decomp.) with preliminary darkening at  $143^\circ$ .

Reduction of petaline chloride with zinc and dilute

hydrochloric acid, similarly gave dihydropetaline chloride.

Dihydropetaline chloride is very soluble in water, but insoluble in ether and chloroform, and could not be crystallised. It again behaved as a pseudo-quaternary

base, showing that the  $\begin{array}{c} | \\ -N = C \\ | \end{array}$  group was not concerned in the reduction.

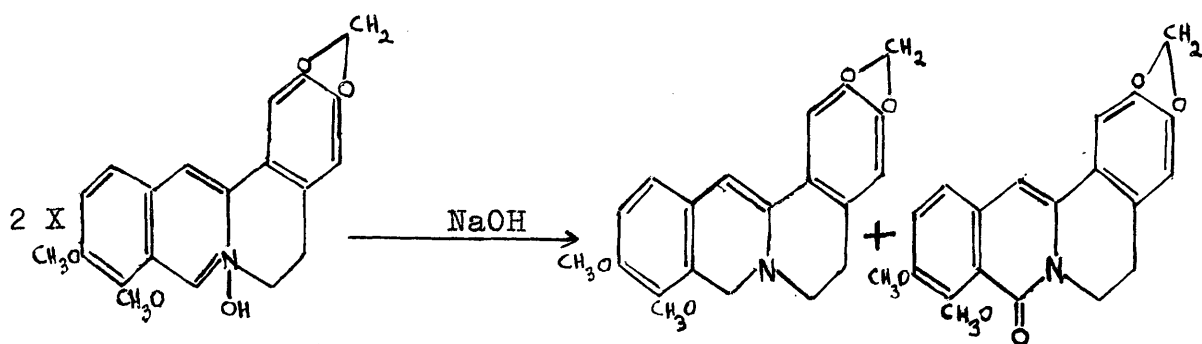
The change in direction of the rotation shows that reduction has affected an asymmetric centre in the molecule.

#### The action of alkali on petaline chloride.

In various attempts to isolate the free base from petaline chloride, it was found that treatment with sodium hydroxide solution (20%) gave a dark, brownish-red solution, from which no ether-, or chloroform-soluble base could be extracted. Evaporation of the solution to dryness gave an almost black resin, which could not be crystallised. Treatment of petaline chloride with an equivalent amount of barium hydroxide, similarly did not produce an ether-, or chloroform-soluble base. However, on adding excess of barium hydroxide and allowing the solution to stand for 8 to 12 days, it was found that about 15 to 20% of the base could now be extracted with chloroform. This amount was increased to 30% by evaporating a hot aqueous alkaline solution to dryness under

reduced pressure, and extracting the residue with chloroform. The chloroform extracts were thoroughly washed with water and dried. Evaporation to dryness and recrystallisation from aqueous ethanol gave a base as almost colourless needles m.p. 117.5-118.5°. This base had an ultra-violet absorption spectrum similar to that already found for leonticine, and it gave a picrate, melting with decomposition at 172.5-173.5°. The base and picrate obtained showed no depression when mixed with leonticine and leonticine picrate. The base also gave a reineckate, m.p. 166-167° (decomp.), which when chromatographed on paper, as before, with leonticine reineckate, showed both reineckates to have identical  $R_F$  values.

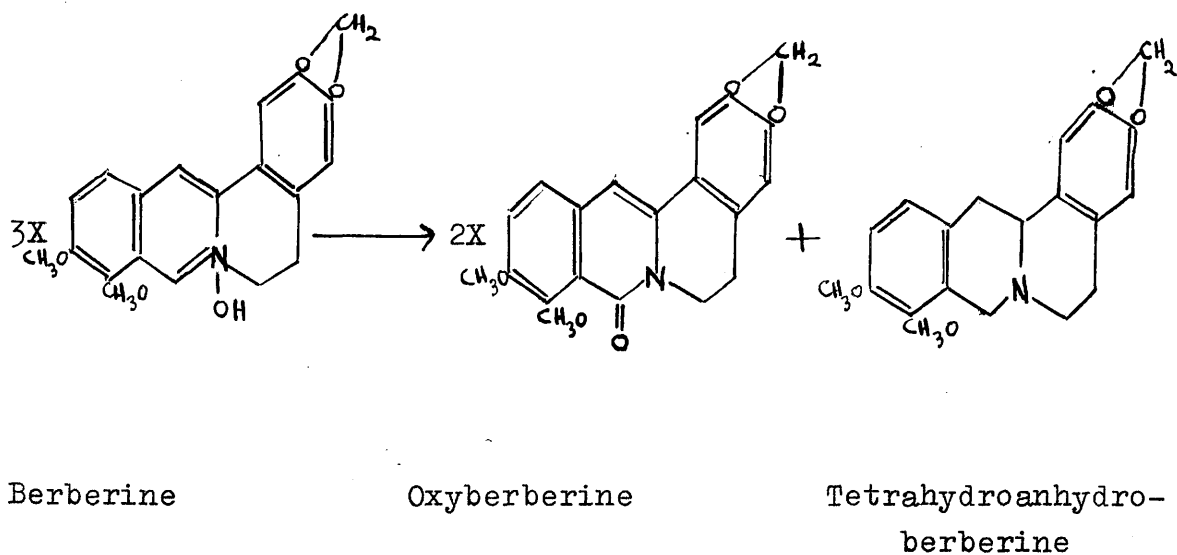
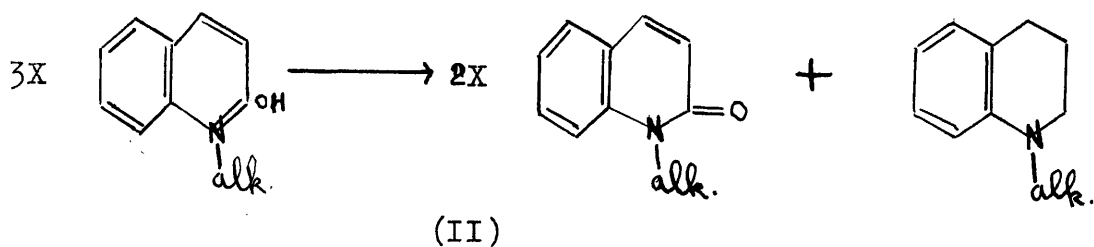
Gadamer<sup>(112)(113)</sup>, observed that treatment of berberine with concentrated caustic soda solution produced a mixture (I) of what he called dihydroberberine and oxyberberine. Perkin<sup>(114)</sup> suggested, that as dihydroberberine was not a direct reduction product of berberine, but was derived from the alkaloid by reduction, and simultaneous removal of the elements of water, that this compound should therefore be named dihydroanhydroberberine. Dihydroanhydroberberine, being a tertiary base, differed from berberine (ammonium form) in being soluble in ether.

Berberine (ammonium  
form)Dihydroanhydro-    Oxyberberine  
berberine

(I)

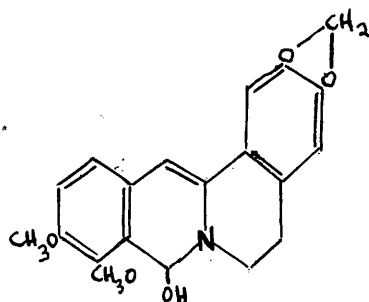
Perkin<sup>(114)</sup> suggested that the reaction with alkali was analogous to the Cannizzaro reaction, in which benzaldehyde is converted to benzyl alcohol and benzoic acid.

Faltis<sup>(115)</sup>, however, has suggested that the reaction is analogous to that between quinoline methiodide and alkali (II), and that the products formed (III) are oxyberberine and tetrahydroanhydroberberine (dl-canadine), although his tetrahydroanhydroberberine was very impure and easily regenerated berberine.



(III)

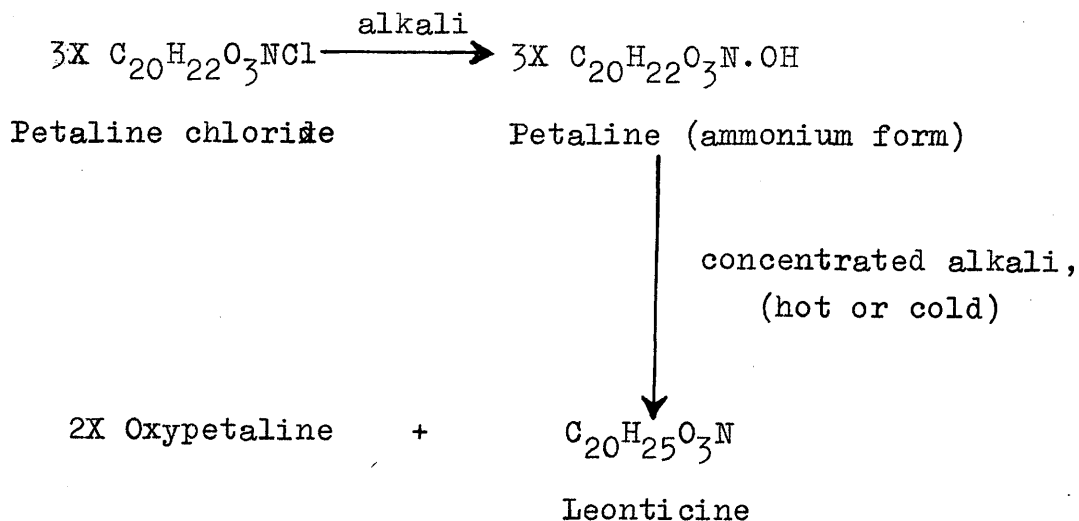
Two forms of berberine are known, the ammonium form (I) and the carbinol form (IV) <sup>(116)</sup>



(IV)

Tinkler<sup>(117)</sup> has observed that ordinary berberine and its salts show the same ultra-violet absorption spectra, whilst the carbinol form (Gadamer's<sup>(112)(113)</sup> so-called berberinal, and Tinkler's<sup>(117)</sup> berberinol) show an absorption spectrum almost identical with that of Freund and Beck's<sup>(118)</sup>  $\alpha$ -methyldihydroberberine, which would appear to be a derivative of the carbinol form of berberine. Further, the absorption spectrum of the hydro-product formed by the action of alkalis on the carbinol form of berberine, is similar to the carbinol form, and is quite distinct from that of tetrahydroanhydroberberine, so that these observations lend no support to Faltis' suggestion.

Strong alkali converts petaline into leonticine which from its empirical formula  $C_{20}H_{25}O_3N$  would appear to be tetrahydroanhydropetaline. Its conversion in approximately 30% yield suggests that decomposition follows a course similar to the action of alkali on quinoline methiodide<sup>(115)</sup>. The formation of a carbonyl compound as the other product of the reaction (V), has been demonstrated by the isolation of a crystalline 2.4.dinitrophenylhydrazone, though the formation of such derivatives would imply that the carbonyl group is not adjacent to the nitrogen (i.e. not an amide group) as in oxyberberine.



(V)

It is possible that leonticine does not occur in the root-tubers of L. leontopetalum, but is formed by the rather prolonged action of alkali (10%) on petaline during the extraction procedure, which may account for the very low yield of leonticine obtained.

#### Oxidation of petaline chloride.

Mild alkaline permanganate oxidation on petaline chloride, by the method of Perkin<sup>(119)</sup> gave two fractions, one soluble in sodium carbonate solution and the other insoluble. The first substance was obtained in very small amounts as a dark-brown, sticky, non-nitrogenous acid fraction, which from its melting point, 196-236° was obviously a mixture. Recrystallisation of the acid fraction from

ethanol (charcoal) gave only a trace of a colourless, amorphous, solid m.p. 194-206°, which was considered not to be homogeneous, and, unfortunately, the amount obtained was insufficient to permit further analysis. The alkali-insoluble portion of the oxidation product was obtained as a pale-brown, sticky, solid, m.p. 110-130°, which gave the reactions of an alkaloid. The product was again, most probably, a mixture, and was insufficient in quantity for further investigation.



The ultra-violet absorption spectra of petaline chloride, dihydropetaline chloride, tetrahydroanhydropetaline and oxypetaline.

The ultra-violet absorption spectrum of petaline chloride, in water, showed three maxima, as distinct peaks at 224 m $\mu$ . ( $\epsilon$  , 20,576), at 280 m $\mu$ . ( $\epsilon$  , 11,600) and a slight inflection at 328 m $\mu$ . ( $\epsilon$  , 334) as shown in Fig.1.

Dihydropetaline chloride in water showed the same two peaks, at 224 m $\mu$ . and 280 m $\mu$ ., although the intensity at 224 m $\mu$ . was slightly reduced ( $\epsilon$  , 15,364), as shown in Fig.1. The third peak, however, was absent, there being no absorption above 290 m $\mu$ . This is in agreement with the results of Feist, Awe and Etzardt<sup>(120)</sup> who found that the dihydro-products produced on reduction (zinc/acetic acid) of Calumba alkaloids (Jatorrhizine, Palmatine and Columbamine), had no absorption above 305 m $\mu$ ; and the absorption curves had maxima at 280 m $\mu$ .

Spectrophotometric examination of tetrahydroanhydropetaline (leonticine), in ethanol, showed a strong band at 218 m $\mu$ . ( $\epsilon$  , 25,957), with a second broad, but intense band at 296 m $\mu$ . ( $\epsilon$  , 25,538), but no absorption above 300 m $\mu$ . (Fig.2). The slight shift in the maxima in the lower wave-length peak of petaline chloride, from 224 m $\mu$ . to 218 m $\mu$ ., may be due to solvent effects, which have been

observed with isoquinoline alkaloids<sup>(121)</sup>. The absorption spectrum obtained for tetrahydroanhydropetaline (leonticine) is in agreement with the observations of Feist, Awe and Etzardt<sup>(120)</sup> on the Calumba alkaloids, in that the tetrahydroanhydro bases showed a broad band at 290 m/ $\mu$ ., and no absorption above 315 m/ $\mu$ ., as distinct from the band at 265 m/ $\mu$ ., in the parent alkaloids.

The curve of oxypetaline chloride similarly showed two distinct peaks at 226 m  $\mu$ . ( $E_{1\text{cm.}}^{1\%}$  1,522 ) and 280 m/ $\mu$ . ( $E_{1\text{cm.}}^{1\%}$  414)(Fig.3) and like oxyberberine<sup>(120)</sup> showed an increase in the intensity of the 280 m/ $\mu$ . peak.

According to Gadamer<sup>(122)</sup>, certain families of plants yield alkaloids of characteristic chemical types. It is possible, therefore, to speculate as to the chemical nature of the constituent alkaloids from different, but related, species. In the Berberidaceae, the majority of the alkaloids belong to the isoquinoline group, and berberine itself is very widely distributed<sup>(123)</sup>. However, the absorption spectrum of petaline chloride differs from that of berberine hydrochloride (Fig.4), and the spectra of petaline chloride, and the derivatives described above, show no specific resemblance to those of the numerous isoquinoline alkaloids discussed by Steiner<sup>(121)</sup>, except that of hydrocotarnine (Fig.5)<sup>(121)</sup>, a reduced isoquinoline alkaloid, which does resemble the spectrum of dihydropetaline chloride.

The ultra-violet absorption spectrum of petaline chloride similarly does not resemble those of jatorrhizine and palmatine, (Fig.6). However, as the spectra of dihydropetaline, and oxypetaline, chlorides, and tetrahydro-anhydropetaline do resemble the corresponding derivatives of both the Calumba alkaloids<sup>(120)</sup> and berberine<sup>(120)</sup>, it is reasonable to assume that petaline probably also belongs to the isoquinoline group of alkaloids.

# Ultra-violet absorption spectra

Figure 1

— Petaline chloride  
 --- Dihydropetaline chloride

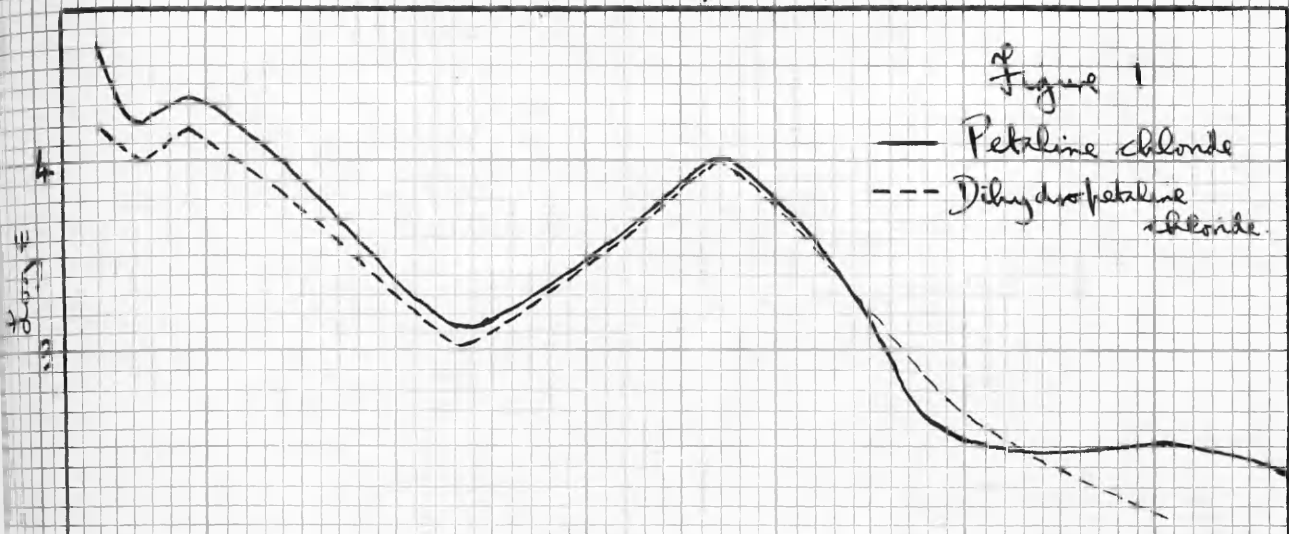


Figure 2

Leonticine  
 (Tetrahydroanhydropetaline.)

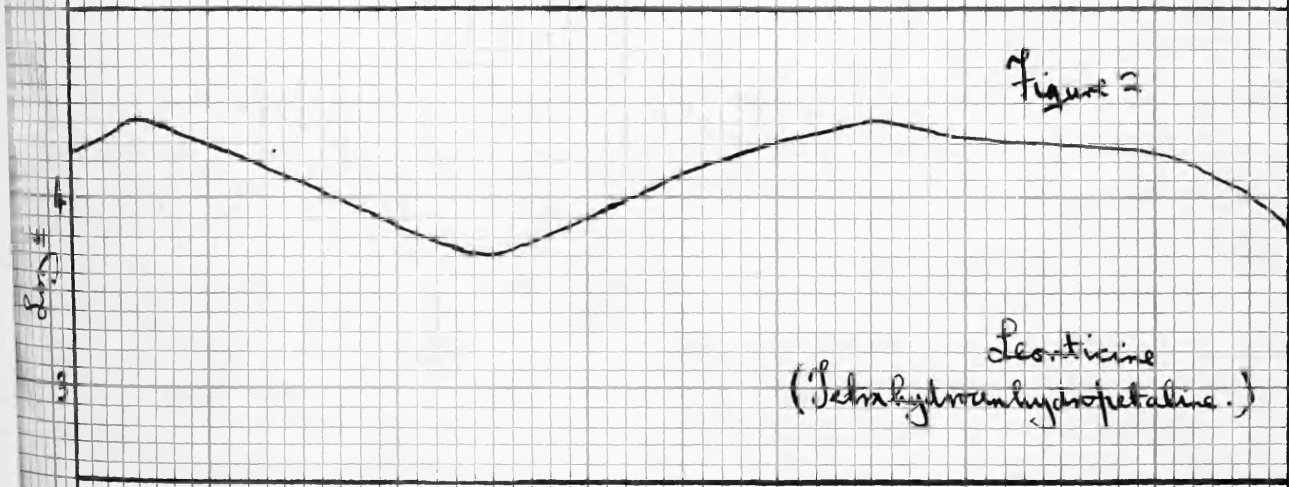
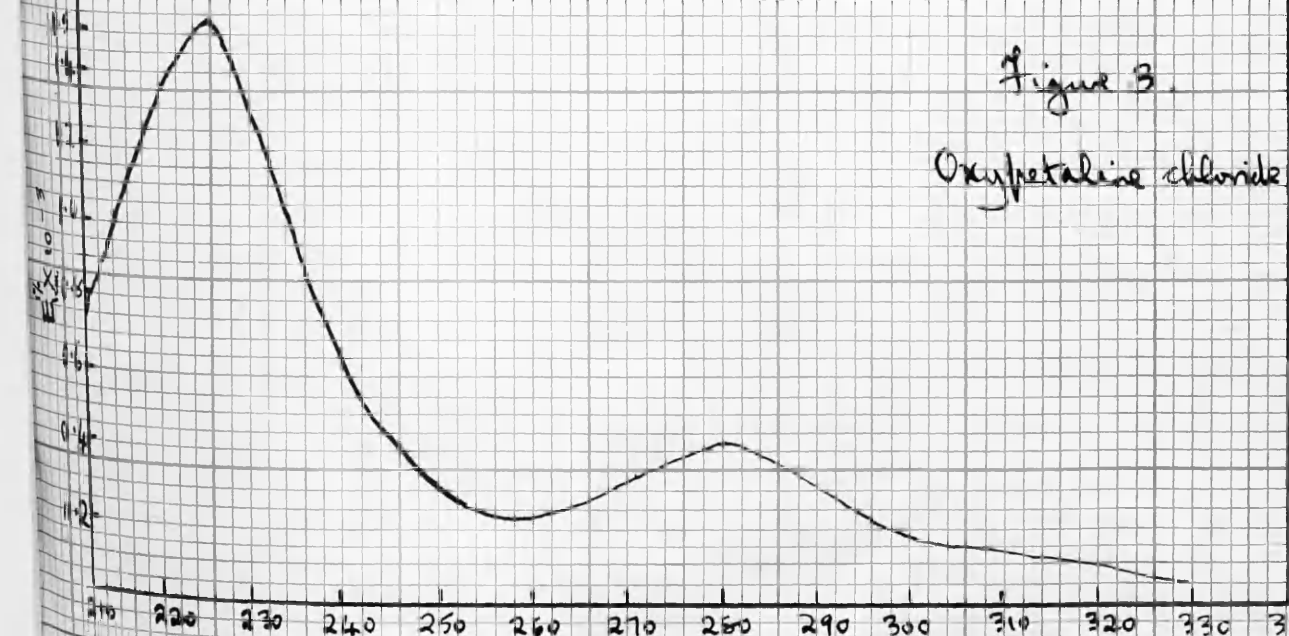


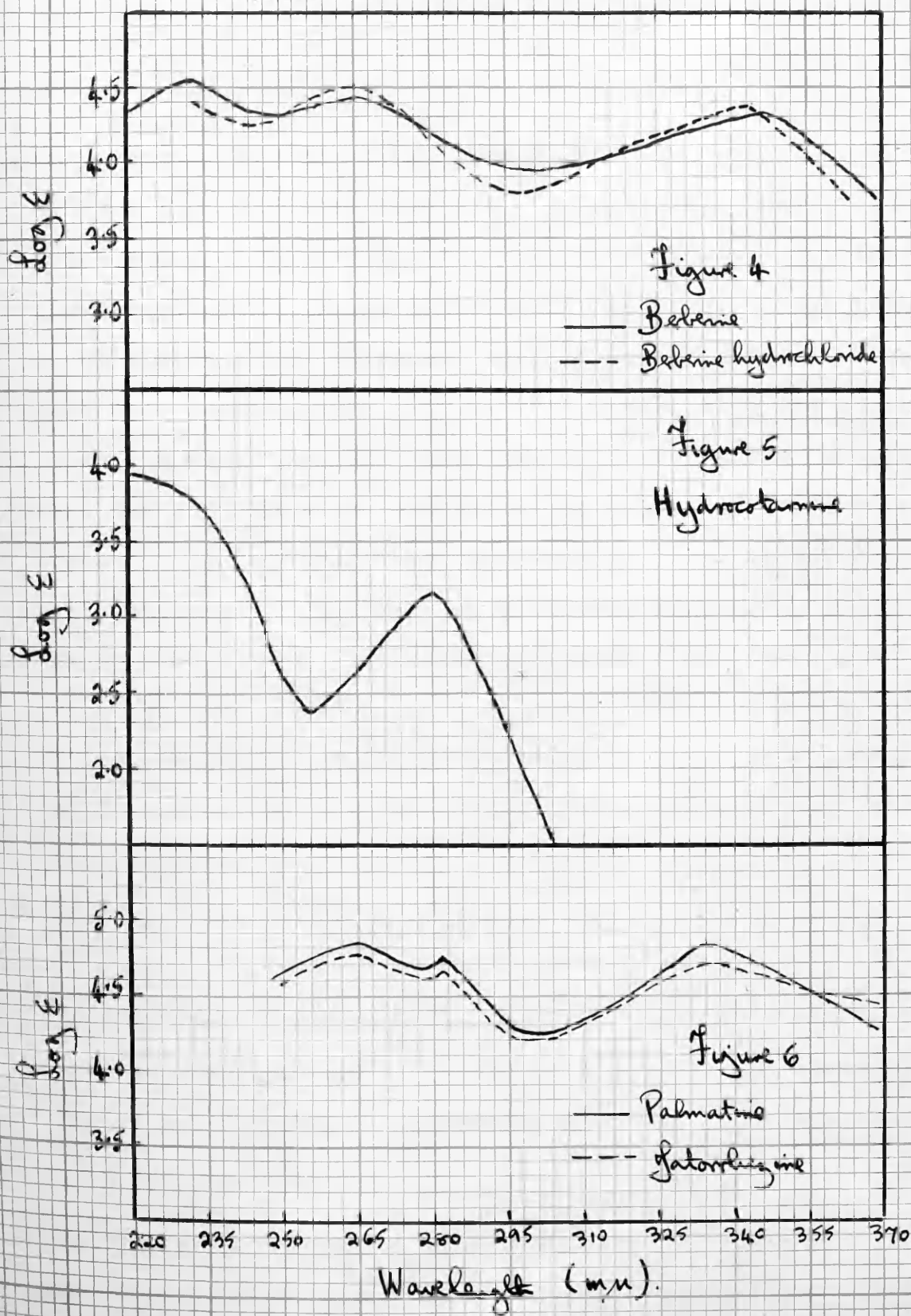
Figure 3

Oxypetaline chloride



Wavelength (mμ).

# Ultra-violet absorption spectra.



LIGHT PETROLEUM EXTRACT OF THE  
ROOT-TUBERS OF L. LEONTOPETALUM

As a preliminary step, the dark-brown, semi-solid extract was subjected to steam distillation. Only a very small quantity of volatile oil was obtained, which possessed a pleasant odour, characteristic of the drug, though insufficient was obtained to permit further investigation. The residual, non-volatile fat, was hydrolysed with 20% ethanolic potassium hydroxide<sup>(124)</sup> and unsaponifiable matter extracted from the soap solution with ether as a yellow viscid oil, (fraction I), which on cooling deposited a small amount of solid material. The soap solution was acidified, and the precipitated fatty acids extracted with light petroleum, (fraction II). The aqueous liquid contained a small amount of ether-soluble material, which was obtained as a brownish-yellow solid, and was not investigated further. The acid, aqueous liquid was reserved for future examination, (fraction III).

EXAMINATION OF THE UNSAPONIFIABLE MATTER (FRACTION I)

The fraction was redissolved in petrol, and chromatographed on a column of alumina to give the fractions shown in Table 6, (page 76).

Fraction A, a colourless oil, deposited crystalline material on cooling, which crystallised from ethyl acetate as beautiful,

Table 6.

Fraction	Volume of Eluate, ml.	Eluant	Weight of Residue (g.)	Remarks
A.	1050.	Petrol	0.57	Colourless oil, deposits crystals on cooling.
B.	1100	(Petrol 98 Benzene 2)	0.50	Colourless oil, deposits crystals on cooling.
C.	1000	(Petrol 70 Benzene 30)	0.094	A pale yellow oil, gives semi-solid on cooling.
D.	1600	Benzene	0.03	Pale yellow oil, on cooling deposits trace of solid.
E.	1000	(Benzene 99.5 Ethanol 0.5)	0.023	Yellow oil, on cool- ing deposits a trace of crystals.
F.	1500	(Benzene 99 Ethanol 1)	3.26	A deep yellow oil, gives a solid on cooling.
G.	1000	(Benzene 99 Ethanol 1)	0.69	A deep yellow oil, gives a semi-solid on cooling.
H.	1000	(Benzene 70 Ethanol 30)	0.019	A yellowish-brown semi-solid.
I.	1100	Ethanol	0.3	Pale brown semi-solid.

soft, lustrous plates. Unfortunately these crystals formed a wax-like cake when filtered. This difficulty was overcome by crystallising the material from ethanol, to give soft, lustrous plates, which did not coalesce on filtering, m.p. 63-64°. The substance contained only carbon and hydrogen, was fully saturated, and appeared to be a long-chain paraffin. This type of compound has been shown to occur in a great many waxes<sup>(125)(126)(127)</sup>, and Chibnall and his colleagues<sup>(127)(128)(129)(130)</sup> who have made a study of these compounds, state that such naturally occurring paraffins are usually mixtures, the components of which contain an odd number of carbon atoms. Evidence based on crystal spacing data and a standardised melting point determination must be studied before a given sample can be declared a single substance, or a mixture. Mixed melting point determinations are also valueless, since the paraffins do not depress the melting points of each other. From its melting point the paraffin isolated may possibly be n-nonacosane  $C_{29}H_{60}$ , m.p. 63.6°, but this could not be confirmed by microanalysis, and lacking other evidence this conclusion must be accepted with reserve.

The bulk of fraction A could not be crystallised. It was non-volatile, was fully saturated, and although possibly a mixture of hydrocarbons, was not examined further.

Fraction B, was eluted from the column with light petroleum



containing 2% of benzene, as a colourless oil, from which a crystalline mixture was obtained. Fractional crystallisation from ethanol or ethyl acetate, failed to separate the mixture, but maceration with light petroleum removed a small amount of a waxy solid, m.p.  $63^{\circ}$ , which was probably identical with the above paraffin. The light petroleum insoluble material was recrystallised from ethyl acetate to give glistening colourless crystals, m.p.  $77-78.5^{\circ}$ . The compound was fully saturated, and analysis showed it to have an empirical formula  $C_{26}H_{54}O$ . This tentative identification as ceryl alcohol, was confirmed by preparation of ceryl acetate, m.p.  $60.5-62.5^{\circ}$  identical with that of genuine ceryl acetate. Insufficient material was obtained to undertake oxidation of the alcohol to the corresponding acid. The term ceryl alcohol has been used instead of the chemical name, n-hexacosanol, since according to Chibnall<sup>(130)</sup>, most of the supposedly pure natural alcohols are mixtures of components which possess an even number of carbon atoms. The possibility of isolating the individual components of such mixtures is considered to be remote<sup>(131)</sup>. Most of fraction B could not be crystallised, was fully saturated and was considered to be a mixture of paraffins and long chain alcohols, hence it was not examined further.

Fractions C, D and E, failed to deposit significant amounts of crystalline material, and were not examined further.

Fraction F, a deep yellow oil, deposited crystals on cooling, which after recrystallisation from methanol had a constant m.p. of 155.5-156.5°, and gave colour reactions indicative of sterols. Analytical data on the sterol, its acetate, and benzoate, indicated an empirical formula  $C_{29}H_{46}O$ . Reinitzer<sup>(132)</sup>, however, first called attention to the futility of basing the empirical formula of sterols simply on such analyses, and indeed in this case the analytical figures could be applied to various homologues of the  $C_{(29)}$  type. However, the best fit is given by the empirical formula  $C_{29}H_{46}O$ .

The sterol gave positive Liebermann-Burchard<sup>(133)(134)</sup> Salkowski<sup>(135)</sup> and Tortelli-Jaffé<sup>(136)(137)</sup> reactions. The presence of a 3- $\beta$ -hydroxyl group was inferred by the production of an insoluble digitonide from the sterol. An attempted purification of the sterol via its acetate, gave the original sterol. Microhydrogenation of the sterol with a platinum oxide catalyst in neutral solution, showed two readily reducible double bonds to be present. Treatment of the sterol acetate with bromine in glacial acetic acid did not precipitate an acetate bromide, the solution turning pale green, indicative of a  $\Delta^7$ -stenol. Addition of water to

the above solution caused the deposition of a very small amount of an almost colourless crystalline bromosterol acetate, m.p. 228-230°. According to Rosenthaler<sup>(138)</sup> the acetate bromide which separates on addition of bromine belongs to the stigmasterol group, whereas that separated by water belongs to the group of sitosterols. Thus it may be that a small amount of a sitosterol is present in the sterol, although it should be noted that decomposition had occurred in the above reaction.

Bergmann<sup>(139)</sup> has classified sterols into six groups according to the specific rotations of the sterols. All sterols having a specific rotation between -20° and +10° are classified in Group III, and Bergmann states that all these sterols possess a double bond in the 7:8 position.  $\Delta^7$ -sterols give positive Liebermann-Burchard, and Tortelli-Jaffé reactions, the latter being specific for steroids with a double bond attached to C<sub>(8)</sub> in the sterol molecule. Of considerable value in recognising sterols belonging to this group is the fact that the sterols melt significantly lower than their acetates, whereas the reverse is true of most of the other steroids<sup>(139)</sup>. The sterol isolated had a m.p. 155.5-156.5° whereas its acetate melted at 159-160°. In addition molecular rotation differences of  $\Delta^7$ -sterols and their acetates are  $-15 \pm 15$ <sup>(140)</sup> (found: +7.6) and of the  $\Delta^7$ -sterols and their benzoates  $+20 \pm 14$ <sup>(140)</sup> (found: +33.6).

Thus it would appear that the phytosterol is a  $\Delta^7$ -stenol; however, Elsevier<sup>(141)</sup> does not record a sterol corresponding to the phytosterol isolated. Thus the phytosterol isolated has not been named as it is probably a mixture of  $\Delta^7$ -stenols (stigmasterols) and may also contain some sitosterol. Lack of material has prevented any further elucidation of the structure of the phytosterol.

EXAMINATION OF THE FATTY ACIDS, FRACTION II.

This fraction was separated into saturated, and unsaturated acids, by the method of Twitchell<sup>(142)</sup>, as modified by Hilditch<sup>(143)</sup>.

The lead salts of the saturated acids were filtered, decomposed with acid, and the free acids converted to the methyl esters and fractionally distilled to give the fractions indicated in Table 7. These fractions were shown to contain no unsaturated fatty acids (iodine value, Table 7) and saponification of the fractions indicated the presence of a C<sub>(16)</sub> acid, fraction (a), and a C<sub>(18)</sub> acid, fraction (b), whilst the non-distillable residue indicated the presence of acids with twenty-four or more carbon atoms. Methyl palmitate was confirmed in fraction (a) by its melting point, equivalent weight, and hydrolysis to palmitic acid, m.p. 60-61°, undepressed on admixture with genuine palmitic acid. A small amount of an acid of high melting point (99-101°) was obtained from fraction (a), which did not correspond to any of the known fatty acids, and on saponification was shown to have an equivalent 351.5, which again did not correspond to that of any known fatty acid. Insufficient material was available to identify this fraction and it was not investigated further.

Methyl stearate was confirmed in fraction (b) by the

melting point of its ester, equivalent weight and hydrolysis to stearic acid, m.p. 67-68.5°, undepressed on admixture with genuine stearic acid.

The non-distillable residue gave an equivalent of 385.3 for the methyl ester, and yielded a mixture of acids, m.p. 73.5-77.5°. This acid mixture could not be separated by crystallisation. The non-volatility of the ester indicates a higher fatty acid ester such as C<sub>(22)</sub>, C<sub>(24)</sub>, C<sub>(26)</sub> or higher, the methyl esters of which are regarded as non-distillable<sup>(144)</sup>, although Williams<sup>(145)</sup> found that methyl lignocerate (C<sub>(24)</sub> acid ester) could be distilled at 190-198°/3 mm.Hg. The methyl ester residue in this work could not be distilled at even higher temperatures, and it was considered that the material might be methyl cerate, which is rarely found in fats, but which is the chief component of many plant waxes<sup>(127)(128)</sup>. Cerotic acid is now recognised to be a mixture of several n-aliphatic acids of even-numbered series, and is not solely n-hexacosanoic acid<sup>(146)</sup>. A normal fatty acid cannot be considered pure unless it has the correct melting point, and correct acid value, and gives both of two characteristic X-ray spacings<sup>(146)</sup>. Cerotic acid has a m.p. of 82°, whereas the acid mixture isolated had a m.p. 73.5-77.5°, mixed m.p. with genuine cerotic acid was 72.5-76.5°, though this depression may not be really significant, as, according to Hilditch<sup>(147)</sup>, the melting points, and mixed melting

Table 7.

Fraction	Colour	Bath temp.	Pressure mm. Hg.	Weight (g.)	E.W.	I.V.	m.p.
(a)	Colourless	220-228°	2	1.6358	274.4 <sup>✓</sup>	0	25-28°
(b)	Colourless	228-250°	2	0.3622	304.18 <sup>✓</sup>	0	37.5-39°
(c)	Brown residue	-	-	0.1912	385.3 <sup>*</sup>	-	58-61°

<sup>✓</sup> C<sub>17</sub>H<sub>34</sub>O<sub>2</sub> requires : E.W. 270, m.p. 29.5-30.5°

<sup>✓</sup> C<sub>19</sub>H<sub>38</sub>O<sub>2</sub> requires : E.W. 298, m.p. 38.5-39.5°

<sup>\*</sup> C<sub>25</sub>H<sub>50</sub>O<sub>2</sub> requires : E.W. 382, m.p. 58°

C<sub>27</sub>H<sub>54</sub>O<sub>2</sub> requires : E.W. 410, m.p. 63°

Table 8.

Fraction	Colour	Bath temp.	Distillation temp.	Pressure mm. Hg.	Weight (g.)	E.W.	I.V.
(d)	Pale yellow	245°	179-184°	5	1.9778	293.84 <sup>✓</sup>	121.43 <sup>✓</sup>
(e)	Pale yellow	250°	184-188	5	0.31	293.6 <sup>*</sup>	98.36 <sup>*</sup>
(f)	Dark brown	-	-	-	1.60	-	-

<sup>✓</sup> C<sub>19</sub>H<sub>34</sub>O<sub>2</sub> requires : E.W. 294.402, I.V. 172.41

<sup>\*</sup> C<sub>19</sub>H<sub>36</sub>O<sub>2</sub> requires : E.W. 296.418, I.V. 85.63

points, of acids of molecular weight above  $296^{\circ}$ , are unreliable as a criterion of purity. A further observation by Hilditch<sup>(148)</sup> that plants of the same family tend to have common fatty acids, and the fact that Power and Salway<sup>(28)</sup> found Caulophyllum thalictroides to contain a mixture of palmitic, stearic and cerotic acids, provides some support for this identification as "cerotic acid". Nevertheless the wide range in melting point ( $4^{\circ}$ ) does suggest that the substance is in fact a mixture of two or more acids, of the cerotic type.

Unsaturated acids were recovered from the soluble lead salts and converted to the methyl esters. These esters were fractionally distilled as before to give the results summarised in Table 8. Linoleic acid was confirmed in fraction (d) by preparation of crystalline tetrabromostearic acid, m.p.  $115^{\circ}$ , undepressed on admixture with genuine tetrabromostearic acid prepared from linoleic acid. The mother liquors from this fraction gave a mixture of crystals which, however, could not be separated, and were not further investigated. Calculation of the ratio of component acids in fraction (d) from the iodine value and equivalent weight<sup>(149)</sup>, having assumed that only  $C_{(18)}$  acids were present in the fraction, suggested that this fraction was a mixture of oleic and linoleic acids (7:5). A similar calculation on fraction (e) indicated it to be a mixture of oleic and linoleic acids



(6.02 : 1). Oxidation of a portion of fraction (e) did not give a crystalline derivative, an oily solid of indefinite melting point being obtained. Lapworth and Pearson<sup>(150)(151)</sup> say that where a fraction consists mainly of oleic acid, it can be separated from the other acids accompanying it by preparation of the barium salt, which is purified, and decomposed to give after distillation pure oleic acid. Application of this procedure to fraction (e) gave an acid, iodine value 91.68, (calculated for 90.5 for pure oleic acid); however, owing to the small amount available it was not distilled, but was considered pure enough to be directly oxidised by the method of Scanlan and Swern<sup>(152)</sup> to give dihydroxystearic acid, m.p. 90-91°, undepressed on admixture with authentic dihydroxystearic acid from oleic acid, m.p. 88-90°. The non-distillable residue, (Table 8), was not examined further. The amounts of the component fatty acids present in the total fatty acid mixture obtained from the root-tubers of L. leontopetalum are shown in Table 9.

Table 9.

Component fatty acids per cent (weight) of total fatty acids						
Oleic	Linoleic	Residue	Palmitic	Unknown	Stearic	Cerotic
23.45	14.45	26.44	22.84	5.14	5.98	1.7

Fraction III. Glycerol was confirmed by evaporation of this fraction, when the residue gave the colour reactions<sup>(153)</sup> of glycerol and gave a crystalline tri-p-nitrobenzoate, m.p. 192-193<sup>0</sup>, undepressed on admixture with genuine glyceryl tri-p-nitrobenzoate. The acids are probably present mainly as the glyceryl esters.

## **EXPERIMENTAL**

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M.p.'s are uncorrected. Rotations were determined in absolute ethanol (unless otherwise stated) in a 1-dm. tube. Ultra-violet absorption spectra were determined in absolute ethanol on a Hilger Uvispek photoelectric spectrophotometer, infrared absorption spectra by using a recording double-beam infrared spectrometer, built in this College by Dr.I.A. Brownlee (J.Sci.Instr., 1950, 27, 215). The author is indebted to Mr.Iengar for the infrared measurements, to Mr.W.McCorkindale, Dr.A.C.Syme and Mr.W.Gardiner for the micro-analyses, to the staff of the Chemistry Department for the use of apparatus, and to Prof.D.H.R.Barton for a sample of hederagenin. The light petroleum used boiled over the range 40-60°. The sliced, dried root-tubers of L. leonto-petalum were powdered in a Christy-Norris disintegrator to give a No.80 powder, and it is this powder that is referred to as "the drug".

PRELIMINARY EXAMINATION OF LEONTICE LEONTOPETALUM.Microsublimation in vacuo.

The drug (3g.) was heated in Tiedermann's apparatus<sup>(154)</sup>, 3.1mg. of a brownish-yellow oily material sublimed, which was not soluble in cold, but readily soluble in hot, ethanol, to give a slightly alkaline solution (pH, 7.2, B.D.H. Universal Indicator Papers). The oil was very slightly soluble in dilute hydrochloric acid, the acid solution giving a faint precipitate (alkaloid) with Mayer's reagent. With aqueous potassium hydroxide (20%), the sublimate gave no red colour, even on warming.

Extraction of the drug with warm water (50°).

The drug (5g.) was warmed with water (30ml.) at 50° for  $\frac{1}{2}$  an hour, cooled, and filtered. The acid filtrate (pH, 6.4), frothed vigorously on shaking, and gave no precipitate on boiling. It did not reduce either Benedict's, or Fehling's solutions and did not react in the Biuret test, or with Millon's reagent, but with Mayer's reagent a copious yellow amorphous precipitate was obtained. The solution (2ml.) with a few drops of ferric chloride solution, gave no blue or black colour, lead acetate solution (10%) gave no precipitate, but with basic lead acetate solution a copious buff precipitate was obtained. The solution (5ml.) when heated on a water-bath

for 1 hour with dilute hydrochloric acid (5ml.) gave a gelatinous precipitate, and the filtrate reduced both Fehling's and Benedict's reagents.

Extraction of the drug with warm 1% sodium carbonate solution.

The drug (5g.) was heated at  $40^{\circ}$  with a 1% solution of sodium carbonate (25ml.). The filtrate gave the following reactions:-

- (a) Shaking produced a copious and persistent froth with a characteristic honeycomb structure. The froth was not destroyed on heating but increased instead, and no precipitate was obtained.
  - (b) The solution gave negative results in both Millon's test and the Biuret reaction.
  - (c) The solution (2ml.) emulsified clove oil (2ml.), giving a coarse unstable emulsion, which broke after 15 minutes.
  - (d) The solution (5ml.), mixed with saline (5ml., 1.8%) and a solution of whole blood in normal saline (5ml.) caused complete haemolysis of the erythrocytes in 10 minutes.
- Controls were carried out with 1% sodium carbonate solution in place of the above solution.

Cyanogenetic test. (155)

The drug (1g.) was put in each of three test tubes and

- (a) treated with water (1ml.) and chloroform (2 drops);

(b) treated with water (1ml.), chloroform (2 drops), and emulsin (0.1g.); and (c) treated with water (1ml.) and dilute sulphuric acid (1ml.). The tubes were corked and a piece of filter-paper, soaked in picrate solution (1% picric acid solution mixed with a warm 10% solution of sodium carbonate), suspended from the cork in each tube. The tubes were kept for 24 hours at 20°; the papers were not coloured red. Tubes (a) and (b) were heated at 60° for 30 minutes without colouration of the papers. Tube (c) when heated to boiling for 5 minutes, gave no colouration of the picrate paper.

#### Extraction of the drug with boiling water.

The drug (10g.) was exhausted by boiling with water (3 portions each of 300ml.) and the extracts filtered whilst still hot. On cooling, the filtrates became gelatinous and gave an intense blue-black colour with iodine solution, and a red precipitate on heating with Benedict's and Fehling's reagents. The combined extracts were evaporated (water-bath) to a syrup, filtered, and whilst still hot, mixed with stirring, with three times its volume of hot ethanol (60%), and again filtered. The filtrate, poured dropwise into twice its volume of ethanol (90%), gave a flocculent, gummy precipitate (0.68g. after drying in vacuo) of crude leontosaponin. Addition of ether to the filtrate caused further precipitation

of crude leontosaponin (0.25g. after drying in vacuo). The saponin fractions were bulked to give crude saponin fraction A, m.p. 190-212° (decomp.).

Extraction of the drug with 1% hydrochloric acid.

The drug (5g.) was heated on a water-bath at 60° with a 1% solution of hydrochloric acid (25 ml.). The filtrate gave heavy precipitates with Mayer's and Dragendorff's reagents, phosphotungstic acid solution, iodine potassium iodide, and picric acid solutions.

The process of Stas-Otto (49).

The drug (50g.) was refluxed with ethanol (250ml., containing 1% tartaric acid) for  $\frac{1}{2}$  an hour. The filtrate was freed from ethanol, and the dark-brown residue (2.3g.) was taken up in warm water (20ml., 60°), cooled, and filtered from a large amount of a brownish-yellow solid. The filtrate was diluted with an equal volume of water, and repeatedly extracted with ether (10 x 25ml.). The ether extracts were evaporated to give 14.2mg. of a pale brownish-yellow solid, fraction (i). The aqueous liquid left after extraction with ether gave reactions for an alkaloid, was made distinctly alkaline with caustic soda (20%) and repeatedly extracted with ether (12 x 25ml.). Removal of the ether gave 0.31g. of a dark-brown semi-solid, fraction (ii). The aqueous liquid,



which still gave alkaloidal reactions, was mixed with saturated aqueous ammonium chloride and repeatedly extracted with chloroform (10 x 25ml.) to give 0.016g. of a dark-brown semi-solid, fraction (iii). The aqueous liquid still gave reactions for alkaloids, was acidified and treated with excess Mayer's reagent to give 0.334g. of a dark-brown amorphous solid, fraction (iv).

Fraction (i) was soluble in water and ethanol. The aqueous solution, treated with a few drops of  $\alpha$ -naphthol in ethanol (20%), and concentrated sulphuric acid run in below the solution, gave a violet ring at the interface of the solutions. The whole solution was similarly coloured on shaking.

Fraction (i) was re-extracted with ether (to remove traces of carbohydrate), and did not reduce either Fehling's or Benedict's solutions. The aqueous solution of fraction (i), (5ml.), heated with dilute hydrochloric acid (5ml.) on a boiling water-bath for 30 minutes, gave a gelatinous precipitate of aglycone and the neutralised filtrate reduced both Fehling's and Benedict's reagents. The sugar solution (5ml.) heated on a boiling water-bath for 30 minutes with phenylhydrazine hydrochloride (0.5g.) and anhydrous sodium acetate (0.75g.), gave a crystalline osazone only on cooling the solution, the crystalline form, although not clearly defined, was indicative of cellobiosazone.

Fraction (ii), was only partly soluble in dilute hydrochloric acid. A dark-brown amorphous, non-alkaloidal, material was insoluble. The acid solution (2 drops) was mixed with each of the following reagents (1 drop), and the colour, and form of the precipitate noted.

- (i) Mayer's reagent - a yellow flocculent precipitate.
- (ii) Dragendorff's reagent - a brown red, granular precipitate.
- (iii) Tannic acid - a faint white, amorphous precipitate.
- (iv) Iodine-potassium iodide solution - a brown amorphous precipitate.
- (v) Picric acid - a dark yellow, sticky, amorphous precipitate.

Fraction (ii) was the only fraction which fluoresced in ultra-violet light, giving a brilliant yellow fluorescence. It also gave the reactions for saponin.

Fraction (iii), gave reactions for alkaloids and saponin.

Again, an appreciable amount of the fraction was insoluble in dilute acid. The acid solution gave the same reactions with alkaloidal reagents as fraction (ii).

Fraction (iv), was shaken with a saturated solution of sodium carbonate, filtered, and the filtrate evaporated under reduced pressure to give a dark-brown, sticky solid, which was alkaloidal, soluble in water, but insoluble in ether.

THE GENERAL METHOD OF EXTRACTION<sup>(56)</sup>

Method (A). The drug (200g.) was extracted in a Soxhlet with (11.) of each of the following solvents in turn.

(1) Light petroleum. The extract on evaporation to dryness gave a yellowish-brown residue (0.444g.), which was re-dissolved in petrol (30ml.), and extracted with a dilute solution of hydrochloric acid (10%, 3 x 20ml.). The acid solution gave no reactions with Mayer's or Dragendorff's reagents, and the slight residue obtained on evaporation failed to react in the concentrated sulphuric acid test for saponin. The petrol, washed thoroughly with water, dried and evaporated, gave a residue which was dissolved in boiling ethanol (90%, 10ml.) and filtered from the insoluble fatty material (0.31g.). On cooling, the filtrate deposited 0.044g., of a waxy solid of low melting point (30-50°). The filtrate on evaporation gave a dark, brownish-yellow residue m.p. 110-129°, (0.0875g.) which gave a positive Liebermann-Burchard reaction.

The fatty material, obtained above, produced a grease spot on paper, and was suspended in water (100ml.), and steam distilled. The distillate had a pleasant aromatic odour, characteristic of that of the drug, but extraction with petrol gave only a trace (1-2mg.) of a pale yellow essential oil.

(2) Ether. This extract gave a yellowish-brown, semi-solid (0.8751g.), which gave reactions for both alkaloids and

saponin. It was dissolved in ethanol (50ml.) and the saponin precipitated with excess ether to give 0.490g. of a pale yellow amorphous solid. The filtrate was evaporated to a brown residue which gave alkaloidal reactions, but did not give the reactions of a phytosterol, or a reducing sugar.

(3) Chloroform. This extract gave a yellowish-brown, semi-solid, (0.568g.) which gave reactions for alkaloids and saponin. The latter was precipitated with ether from alcoholic solution to give 0.426g. of a yellow amorphous solid. The residue gave reactions for alkaloids, but not for a reducing sugar.

(4) Ethanol, extracted 44.6g. of a yellowish-brown, semi-solid, which was re-dissolved in ethanol (250ml.), saponin being precipitated with ether as a yellow amorphous solid (31.78g.), m.p. 212-225° (decomp.). The filtrate gave the reactions for alkaloids, but not of a reducing sugar. It was evaporated to give a dark-brown sticky alkaloid residue, still containing saponin, which could not be recrystallised from ethanol, chloroform, or benzene. The residue was dried in vacuo, dissolved in warm dry ethanol, dry hydrochloric acid gas passed in, and the warm solution mixed with dry ether. On cooling, a gummy dark-brown hydrochloride was deposited, which failed to crystallise.

(5) Ethanol (70%). The drug, removed from the Soxhlet, was

boiled with ethanol (70%) and evaporation of the filtrate gave a brown, semi-solid (2.86g.) which gave reactions for alkaloids, saponins, and a faint precipitate with Benedict's reagent. The residue, redissolved in absolute ethanol, was treated with dry ether to give a yellow saponin, (1.716g.), m.p.201-218<sup>o</sup> (decomp.). The filtrate, on evaporation, gave a dark-brown, sticky alkaloidal residue, which still contained some saponin.

(6) Cold water. The drug was macerated with cold water for 48 hours, and the filtrate evaporated to give a dark-brown, sticky residue (0.98g.) which gave the reactions for alkaloids, saponins, and contained a small amount of reducing sugar. It gave no reaction in either the Biuret, or Millon's, tests. Ether precipitated the saponin as before to give 0.768g. of a yellow amorphous solid.

(7) Boiling water. The drug was boiled with water for 4 hours, and the solution filtered whilst still hot. The filtrate gave faint reactions for alkaloids and saponins, but contained a good deal of starch. On cooling, the solution set to a gel, and gave strongly positive reactions with both Fehling's and Benedict's reagents. Evaporation to dryness gave 15.869g. of a yellow amorphous solid, which gave an intense blue-black colour with iodine solution.

(8) Cold 1% hydrochloric acid. The drug was macerated with this solvent in a sealed vessel for 48 hours. The filtrate, evaporated to dryness on a boiling water-bath, gave a dark-brown sticky residue (0.68g.), which gave only faint reactions for both saponins and alkaloids, but very strongly positive reactions for a reducing sugar. The drug was not examined further.

Method (B). The drug (100g.) was treated successively with the same solvents as in Method (A), first by maceration for 24 hours, and then by percolation to exhaustion with the same solvent. The volumes of solvents used in each case are shown in column 2 of Table 1 (page 20a). In every case cold percolation extracted the same constituents as the hot extractions with the exception that cold 70% ethanol extraction did not release reducing sugars. The extracts were examined qualitatively as in Method (A), but in no case were the constituents isolated as in that method.

ISOLATION OF AN ENZYME<sup>(28)</sup>

The drug (100g.) was macerated with water (containing 0.5% chloroform to prevent mould growth) for 48 hours in a sealed vessel. The filtrate gave a slight reaction for a reducing substance with Benedict's reagent, and was treated with twice its volume of ethanol. The yellowish-grey amorphous solid deposited was rapidly centrifuged from the ethanol, and the solid washed several times with ethanol to remove some yellow colouring matter. The solid was dried in vacuo over phosphorus pentoxide, to give a grey amorphous solid, which did not melt, but began to decompose at  $230^{\circ}$ , giving off a foul odour. It did not give the Biuret, Xanthoproteic, Millons, Sakaguchi's or Ninhydrin, tests for proteins. It gave the reactions of carbohydrates (Molisch's test), reducing sugar (Fehling's and Benedict's tests), and saponin (concentrated sulphuric acid). The solid (0.5g.) was dissolved in water (10ml.) in a collodion membrane, and dialysed for 24 hours in water. The solution no longer reduced Fehling's or Benedict's reagents.

Solutions of Salicin, and  $\alpha$ -methyl D-glucoside, (1%, 5ml.) were each treated with 1ml. of the enzyme solution, shaking well. 1ml. of a solution of a  $\beta$ -glucosidase (1%) obtained by a standard method<sup>(89)</sup> from defatted almond meal was also added to similar solutions of the glucosides (5ml.),

and the solutions placed in a constant temperature bath at 40°. At 10 minute intervals for the first 2 hours, and  $\frac{1}{2}$  hour intervals thereafter, 2 drops of the solution were removed, and tested with Benedict's reagent, to give the results shown in Table 2 (page 22).

The enzyme solution from L. leontopetalum was boiled for 10 minutes, and this solution tested as before to give the results also shown in Table 2 (page 22).



ISOLATION OF LEONTOSAPONIN

Method 1. The drug (400g.), was exhausted by boiling with ethanol (2l.) for 24 hours, the extract filtered whilst still hot and diluted by the addition of excess dry ether, when crude leontosaponin was obtained as a yellow flocculent precipitate, fraction B (45.7g. when dried in vacuo), m.p. 218-226° (decomp.). A further yield of 8.5g. of a dark yellow, amorphous solid, fraction C, m.p. 224-231° (decomp.), was obtained on concentrating the filtrate, and repeating the precipitation with dry ether. Further concentration and precipitation gave a dark yellowish-brown saponin fraction, D, (14.3g.) m.p. 188-194° (decomp.). The filtrate was evaporated to dryness, and the residue reserved for examination of the alkaloids.

Method 2. The drug (400g.) was exhausted by boiling for 24 hours with ethanol 95% (2l.), the extract filtered whilst still hot, and concentrated to a soft extract (75g.). This extract was mixed with water (250ml.) to give a dark-brown solution containing a large amount of suspended yellow material. The solution did not reduce Benedict's solution, and was steam distilled for 3 hours to give 3.7mg. of a pale yellow essential oil. The non-volatile aqueous liquid now reduced both Benedict's and Fehling's solutions, and was repeatedly extracted with hot amyl alcohol (15 x 30ml., 50°)

to give a dark yellowish-brown, amorphous saponin fraction, E, (33g.). The aqueous filtrate was reserved for examination of the alkaloids.

Method 3. The drug (933g.) was exhausted by boiling with absolute ethanol. The ethanolic extract was filtered whilst still hot, and on cooling deposited crude leontosaponin (70g.), as a yellow solid fraction F, m.p.217-221° (decomp.). The filtrate did not reduce Benedict's solution, and was evaporated to give a dark-brown gummy residue (116g.), which after the addition of water (350ml.) and steam distillation for 30 minutes (to give 7.8mg. of essential oil), gave on cooling the solution, a dark yellow amorphous saponin fraction, G (20g.) m.p.212-222° (decomp.). The filtrate reduced both Benedict's and Fehling's solutions, and was repeatedly extracted with hot amyl alcohol (10 x 50ml., 50°), to give a dark yellowish-brown, amorphous saponin fraction H, (62.6g.), m.p.188-220° (decomp.). The filtrate was reserved for examination of the alkaloids.

Method 4. The drug (14.106Kg.) was macerated with light petroleum for 24 hours, and percolated to exhaustion with the same solvent (20l.). Removal of the solvent from the percolate gave 31.1g. of a fat-like residue which was reserved for future examination. The drug was then air-dried, and completely exhausted by refluxing with Industrial

Methylated Spirit (50l.). The filtrate was concentrated to give a dark-brown, soft extract (5.4Kg.). 1.9Kg. of this extract was then redissolved in boiling absolute ethanol (21l.), filtered rapidly, whilst still hot, from a small amount of inert material. On cooling this solution, crude saponin was obtained as a yellow amorphous solid, fraction I, 416.9g (after drying in vacuo), m.p.219-236° (decomp.). The filtrate was again concentrated to a dark-brown semi-solid, water (400ml.) added, and the suspended dark yellow solid filtered to give an amorphous saponin fraction J, 119.12g. (after drying in vacuo), m.p.212-238° (decomp.). The filtrate did not reduce Benedict's reagent, and the aqueous solution was repeatedly extracted with hot amyl alcohol (60°, 25 x 50ml.). The extracts were united, washed with water, dried (sodium sulphate), and the solvent removed under reduced pressure to give a dark yellowish-brown saponin fraction, K, 325.57g. (after drying in vacuo), m.p. 192-206°. The filtrate did not reduce Benedict's reagent and was reserved for examination of the alkaloids.

PURIFICATION OF LEONTOSAPONIN

The fractions obtained were each purified separately as follows:-

Method (1). The fractions, dissolved in boiling absolute ethanol (15 parts), were filtered whilst still hot. About 75% of the saponin was in every case soluble, fraction x. The insoluble portion, fraction y, was soluble in a further 15 parts of boiling ethanol. Both the ethanol solutions obtained in every case were decolourised with activated charcoal, and the saponin recovered by concentrating the solutions, and precipitating with dry ether. Saponin fractions were obtained, varying in colour from pale yellow to yellow, but all melting with decomposition in the range  $224-236^{\circ}$ , and had specific rotations varying between +11 to  $+14.5^{\circ}$ . Excess charcoal caused strong adsorption of the saponin, which was recovered only with difficulty using a large volume of boiling ethanol. Solution of the saponins in methanol, followed by repeated reprecipitation with dry ether failed to remove colouring matter, as did repeated crystallisation from ethanol.

Method (2). The saponin fractions, dissolved in the minimum amount of water, were each dialysed in a collodion membrane for 48 hours in a stream of water. The saponin solutions

were now free of inorganic salts, and were carefully evaporated ( $50^{\circ}$ ) to dryness under reduced pressure, when, in every case, pale buff to pale yellow saponins were obtained, melting with decomposition in the range  $232-236^{\circ}$ .

Method (3).     The Method of Rosenthaler<sup>(62)</sup>. Fraction G, (0.3g.) was dissolved in water (20ml.), mixed with freshly precipitated magnesium hydroxide (1.5g.), and the mixture evaporated to dryness on a water-bath. The pale yellow residue was powdered, refluxed with ethanol (200ml.) for 1 hour, the solution filtered, and the saponin fractionally precipitated with ether. The first two fractions contained inorganic matter, and melted at  $220-230^{\circ}$  (decomp.), and  $221-230^{\circ}$  (decomp.) respectively. The later salt-free fractions (0.03g.) were still pale buff in colour, and melted with decomposition at  $233-238^{\circ}$ .

Method (4).     Preparation of leontosaponin acetate and hydrolysis of this acetate. The partly purified fraction I (1.0g.) now melting with decomposition at  $232-236^{\circ}$ , was refluxed with acetic anhydride (15ml.) on a water-bath until a clear solution was obtained, and thereafter for a further 2 hours. The reaction mixture was poured into ice-water (150ml.), cooled in ice for 30 minutes, stirring well, when a pale yellow sticky acetate separated, which was filtered, and dried in vacuo (0.82g.). The aqueous liquid was extracted

with ether, the bulked ether extracts washed with water, dried (sodium sulphate), and the solvent removed to give a further 0.3g. of acetate. The crude product recrystallised from 60% ethanol (charcoal), gave a colourless, crystalline acetate, m.p.155-156°. This acetate was refluxed with ethanolic barium hydroxide for 30 minutes, treated with solid carbon dioxide until the solution was just acid, and the precipitated barium carbonate filtered off. The colourless filtrate, evaporated to dryness, gave a colourless scale-like saponin, which was recrystallised from dry ethanol to give leontosaponin as a colourless, microcrystalline solid, (0.5g.) m.p.236-238° (decomp.).

Method (5). The saponin fractions were separately triturated in a mortar with a small volume of cold anhydrous ethanol, the yellow solution filtered, and the undissolved solid similarly treated with successive small quantities of ethanol, until the filtrate was colourless. The almost colourless residue was recrystallised from boiling anhydrous ethanol to give in every case leontosaponin as colourless, microcrystalline prisms, m.p.236-238° (decomp.). Leontosaponin is soluble 1 in 30 in cold distilled water, 1 in 15 and 1 in 30, in boiling methanol and ethanol respectively, and 1 in 100 in cold ethanol. The aqueous solution has a pH of 6.8,  $[\alpha]_D^{20} +15^\circ$  (c, 1.0 in distilled water). In the above method of purification of leontosaponin anhydrous conditions were

essential. The combined yellow filtrates, were concentrated to dryness, and the residue treated as before to yield a further small amount of leontosaponin.

Leontosaponin acetate. Leontosaponin (0.76g.) was refluxed with acetic anhydride (10ml.) and sodium acetate (0.56g.) on a water-bath until solution was effected, and thereafter for a further 2 hours. The cooled reaction mixture was poured into crushed ice (2g.), and the mixture cooled in ice for 30 minutes. The solution was carefully neutralised with caustic soda solution and extracted with ether. The bulked ether extracts were washed with water, dried (sodium sulphate) and evaporated to a colourless solid, which was recrystallised from ethanol (60%) as colourless small needles, m.p. 155-156°,  $[\alpha]_D^{20} +19.9^\circ$  (c, 1.0) (Found: C, 55.76; 55.69; H, 6.69; 6.77 per cent.).

LEONTOSAPOGENIN

Hydrolysis of leontosaponin with 4N. acid<sup>(67)</sup>. Crude leontosaponin (1g., fraction E) was dissolved in aqueous (50%) ethanolic hydrochloric acid (4N with respect to HCl, 40ml.), benzene (16ml., previously equilibrated by shaking with an equal volume of 50% aqueous ethanol) added, and the mixture refluxed for 4 hours on a water-bath. The buff coloured, crude sapogenin was filtered, washed with 50% ethanol, and the solid refluxed with benzene (150ml. including the above fraction), and methanolic potassium hydroxide (50ml., 20%) for 1 hour. The benzene solution was separated, and the aqueous layer washed with benzene (50ml.). The bulked benzene fractions were washed with water, dried (sodium sulphate), and the solvent removed to give a yellowish-brown, oily solid (20mg.), m.p. 112-120°. This solid gave positive Liebermann-Burchard and Salkowski reactions. Dilution of the methanolic potassium hydroxide solution with water (50ml.), gave potassium leontosapogenin (0.265g., 26.5% of saponin), an almost colourless crystalline solid (from methanol), which did not melt below 360°.

The dark yellowish-brown sugar solution was neutralised, and reserved for future examination.



Hydrolysis of leontosaponin by the method of Power and Salway<sup>(28)</sup>.

(a) Pure leontosaponin (1.67g.) was dissolved in ethanol (50ml.) containing dilute hydrochloric acid (15ml.), and refluxed on a water-bath for 5 hours with constant stirring. During heating a gelatinous precipitate separated (30 minutes), which on further heating gradually became crystalline. The brown sapogenin obtained was filtered, washed free of acid and dried. Ethanol was removed from the filtrate, water being added to keep the volume constant, when more sapogenin was precipitated. The product (0.4091g., 24.5% of saponin) recrystallised from 90% ethanol (charcoal) gave leontosapogenin as a colourless crystalline solid, m.p. 332-333° (0.3826g., 22.91% of saponin).

The pale yellowish-brown sugar solution was neutralised and reserved as before.

(b) Crude leontosaponin (35g.) was heated with methanol (900ml.) and dilute hydrochloric acid (190ml.) under reflux on a water-bath for 5 hours. After dilution with water (200ml.), methanol was removed by distillation, and the precipitated sapogenin (8.64g., 24.7% of saponin) separated and recrystallised from 80% ethanol (charcoal) to give leontosapogenin m.p. 332-333°, with preliminary slight sintering at 319°.

The sugar solution was neutralised and reserved as before.

Method of choice. Leontosaponin (1.5g.) was heated with methanol (50ml.) and dilute sulphuric acid (15ml.) under reflux on a boiling water-bath for 8 hours. The precipitated sapogenin was isolated and purified as above to yield leontosapogenin, m.p. 332-333° with preliminary sintering at 315-319° (0.4096g.; 29.3% of saponin)  $\epsilon$  2860 at 210 m $\mu$ .,  $[\alpha]_D^{20}$  +79.6° (c, 0.1); +80.4° (c, 1.0 in pyridine).

(Found: C, 76.3; H, 10.1; Equiv. 474.1. Calculated for  $C_{30}H_{48}O_4$  : C, 76.2; H, 10.2 per cent; Equiv. 472.7)

The pale yellowish-brown acid sugar solution was neutralised with a slurry of barium carbonate, and the precipitated barium sulphate filtered off and washed with a little water. The salt free solution was reserved for future examination.

Further hydrolysis experiments with both dilute hydrochloric and dilute sulphuric acids, for periods ranging from 5 to 9 hours gave the percentage yields of sapogenin as shown in Fig.7.

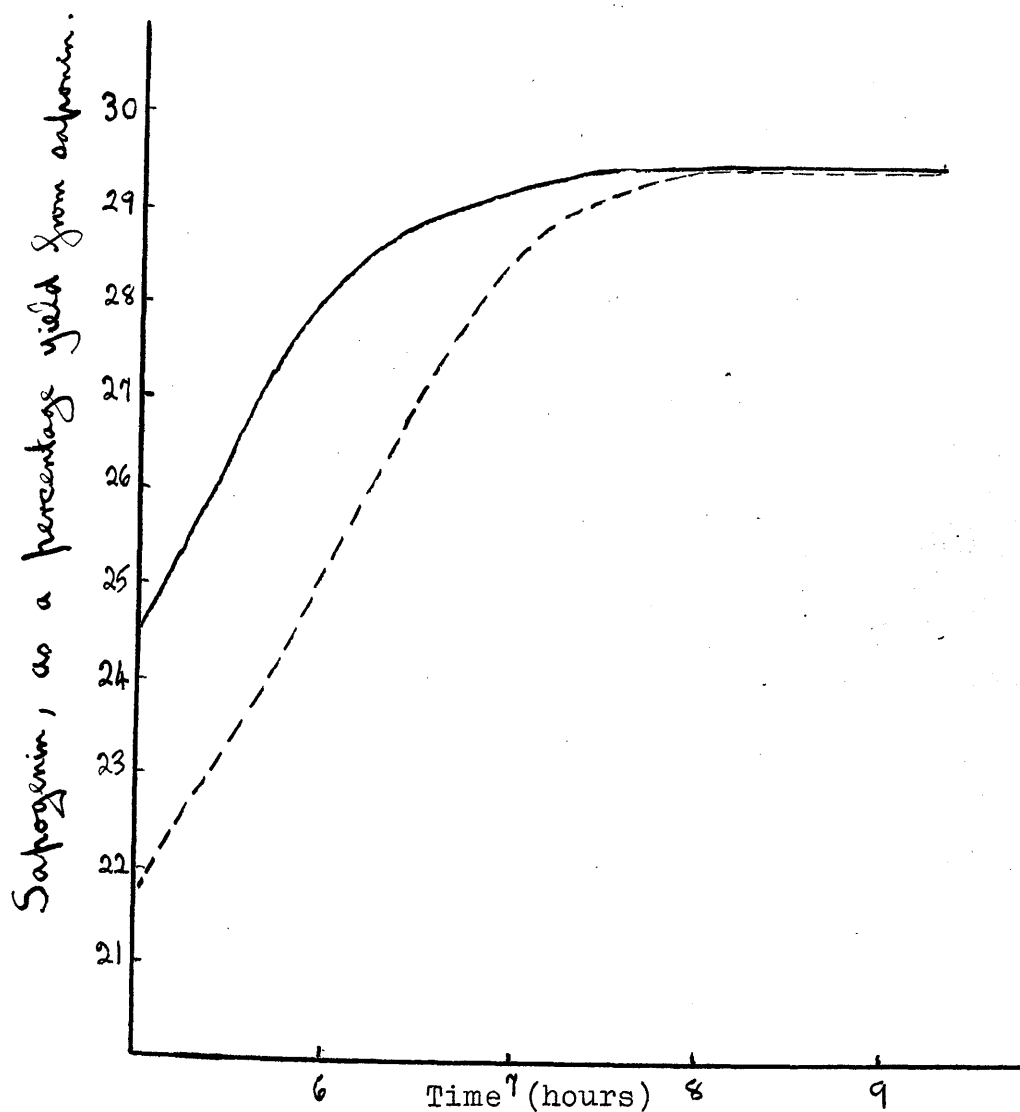


Figure 7. Comparison of the hydrolysis rates of dilute hydrochloric and dilute sulphuric acids on leontosaponin.

--- dilute sulphuric acid; — dilute hydrochloric acid.

Attempted hydrogenation of leontosapogenin. Leontosapogenin (0.2g.) was dissolved in ethanol (80ml.), platinum oxide catalyst (0.0495g.) added, and hydrogenated for 15 hours. There was no uptake of hydrogen, and leontosapogenin was recrystallised from the solution, m.p.  $332-333^{\circ}$ ,  $\epsilon$ , 2860 at 210 m $\mu$ .

Test for unsaturation. Leontosapogenin (2mg.) in 0.05ml. ethanol, gave a pale yellow colour (30 minutes) with a solution of tetranitromethane in carbon tetrachloride.

Isolation of hederagenin from the crude saponin of soap nuts fruits. The crude saponin was hydrolysed with dilute sulphuric acid (8 hours), and the hydrolysate (5g.) purified as before, to give colourless, well-formed rhombic prisms, m.p.  $332-333^{\circ}$ , with preliminary sintering at  $320^{\circ}$ ,  $[\alpha]_D^{20} +79.2^{\circ}$  (c, 0.12). Mixed m.p. with leontosapogenin,  $332-333^{\circ}$ .

Leontosapogenin Diacetate. Leontosapogenin (0.47g.) was refluxed with acetic anhydride (5ml.) on a water-bath for 1 hour, and the reaction mixture poured into ice-water (50ml.) and allowed to stand for 1 hour. The gum which at first separated, gradually hardened, was collected, and recrystallised from methanol (50%) to give colourless stout needles of constant m.p.  $173-174^{\circ}$ , with preliminary sintering at  $157-158^{\circ}$ ,  $[\alpha]_D^{20} +63^{\circ}$  (c, 0.61);  $+66.4^{\circ}$  (c, 2.08 in ethanol 95%)

$\epsilon$ , 2,648 at 209.5 m/ $\mu$ . (Found: C, 73.3; H, 9.6 ,  
Equiv. (by hydrolysis) 553. Calculated for  $C_{34}H_{52}O_6$  :  
C, 73.3; H, 9.4 per cent., Equiv. 556.76.)

Hederagenin Diacetate, prepared as above from pure hederagenin had m.p. 172-173°, with preliminary sintering at 158-159°,  $[\alpha]_D^{20} +63.8^\circ$  (c, 1.0). Mixed m.p. with leontosapogenin diacetate, 172-173°. Jacobs<sup>(77)</sup> gives for hederagenin diacetate m.p. 172-174°,  $[\alpha]_D^{20} +64^\circ$  (c, 1.0).

Leontosapogenin Dibenzoate. Leontosapogenin (2g.) was refluxed with benzoyl chloride (2ml.) and pyridine (20ml.) for 1 hour on a water-bath, and the mixture poured into aqueous sodium bicarbonate. The gummy benzoate was recrystallised from ethanol (60%) to give well-formed prisms of constant m.p. 290-291°,  $[\alpha]_D^{20} +114.5^\circ$  (c, 0.1 in chloroform). (Found: C, 77.4; H, 8.3. Calculated for  $C_{44}H_{56}O_6$  : C, 77.6; H, 8.3 per cent.).

Hederagenin Dibenzoate, prepared from pure hederagenin as above, had m.p. 290-291°,  $[\alpha]_D^{20} +115.2^\circ$  (c, 0.1 in chloroform). Jacobs<sup>(77)</sup> gives m.p. 290-291°, and Power and Salway<sup>(28)</sup> give for caulosapogenin (= hederagenin) dibenzoate,  $[\alpha]_D +115^\circ$ . Mixed m.p. with leontosapogenin dibenzoate, 290-291°.

Leontosapogenin Methyl Ester. Leontosapogenin (1g.) was

shaken with excess diazomethane in ether (80ml.). The ether solution on evaporation gave a crystalline residue, which was recrystallised from aqueous methanol (70%) to give long, slender needles, m.p. 236-237°,  $[\alpha]_D^{20} +74.5^\circ$  (c, 1.018). (Found: C, 76.1; H, 10.3. Calculated for  $C_{31}H_{50}O_4$  : C, 76.2; H, 10.3 per cent.).

Hederagenin Methyl Ester, prepared as above had m.p. 236-237°,  $[\alpha]_D^{20} +75^\circ$  (c, 1.0). Jacobs<sup>(77)</sup> gives for hederagenin methyl ester, m.p. 238-240°,  $[\alpha]_D^{20} +75^\circ$  (c, 1.0). Mixed m.p. with leontosapogenin methyl ester, 236-237°.

Leontosapogenin Methyl Ester Diacetate. Leontosapogenin methyl ester (0.177g.) was refluxed with acetic anhydride (4ml.) for 1 hour on a water-bath, and the reaction mixture poured on to ice. Recrystallisation from aqueous ethanol (60%) gave the diacetate as long needles, m.p. 193-194°,  $[\alpha]_D^{20} +63^\circ$  (c, 0.564). (Found: C, 73.8; H, 10.0. Calculated for  $C_{35}H_{54}O_6$  : C, 73.6; H, 9.5 per cent.).

Hederagenin Methyl Ester Diacetate, prepared from hederagenin methyl ester as above had m.p. 192-193°,  $[\alpha]_D^{20} +62.5^\circ$  (c, 1.0). Mixed m.p. with leontosapogenin methyl ester diacetate, 192-193°. Van der Haar<sup>(72)</sup> gives for hederagenin methyl ester diacetate, m.p. 193°,  $[\alpha]_D^{20} +62^\circ$  (in ethanol).

Acetonyl Leontosapogenin Methyl Ester. A solution of leontosapogenin methyl ester (0.0744g.) in dry acetone (3ml.), with 3 drops of concentrated hydrochloric acid, deposited a mass of lustrous platelets, recrystallised from ethanol, m.p. 250-251°. (Found: C, 77.5; H, 10.3. Calculated for  $C_{34}H_{54}O_4$  : C, 77.5; H, 10.3 per cent.). Concentrated hydrochloric acid turned the crystals yellow, gradually becoming orange. The acetonyl derivative (0.05g.) was refluxed with acetic anhydride (1ml.) and sodium acetate (0.03g.) for 1 hour. No acetate was obtained.

Acetonyl Hederagenin Methyl Ester, prepared from hederagenin methyl ester as above, had m.p. 250-251°, mixed m.p. with acetonyl leontosapogenin methyl ester, 250.5-251.5°.

ISOLATION AND IDENTIFICATION OF THE SUGARS  
FORMED BY ACID HYDROLYSIS OF LEONTOSAPONIN

The neutral sugar solutions (except the salt free sugar solution isolated in the method of choice) were bulked and decolourised with charcoal. The colourless filtrate gave a positive reaction in the Molisch test, reduced Fehling's, Benedict's and Barfoed's reagents, gave a blue-green colour with Bial's reagent (concentrated hydrochloric acid and ethanolic orcinol-ferric chloride), and a cherry-red colour in the aniline test (glacial acetic acid and aniline).

Osazone formation<sup>(156)</sup>. The solution (5ml.) was heated with phenylhydrazine hydrochloride (4g.) and anhydrous sodium acetate (0.6g.), on a boiling water-bath for 10 minutes, when glucosazone, m.p. 201-204°, was deposited as clusters of long slender needles. Further heating of the filtrate for 10 minutes, gave arabinosazone as a tangled mass of crystals m.p. 201-204°, and traces of a third crystalline osazone, which from its appearance may have been cellobiosazone.

Treatment with lead acetate<sup>(157)</sup>. The sugar solution (2ml.) was boiled with a small quantity of lead acetate. Dilute solution of ammonia (5ml.) was added, and the solution reboiled, when a salmon-pink precipitate was obtained.



Potassium Acid Saccharate<sup>(158)</sup>. Evaporation of a portion of the sugar solution (100ml.) to dryness on a water-bath deposited a large amount of salt. The residue, extracted repeatedly with boiling dry ethanol, gave, on evaporation of the ethanol, a sticky residue which contained traces of salt. It was redissolved in boiling ethanol, filtered whilst still hot and re-evaporated. The residue (1g.), was evaporated with concentrated nitric acid (6ml.) on a boiling water-bath until evolution of red fumes had ceased, and the resulting syrup had a permanent yellow colour. This syrup was dissolved in a little water, and powdered potassium carbonate added, warming gently until the solution was neutral. Excess glacial acetic acid was added, and the solution allowed to cool. The crystals formed were filtered, decolourised (charcoal), and recrystallised from water as large colourless rhombic crystals with trapezoidal faces, characteristic of genuine potassium acid saccharate<sup>(158)</sup>.

Silver Saccharate. Potassium acid saccharate (0.1g.) was dissolved in water, neutralised with ammonia, and silver nitrate solution added to give a white characteristic precipitate of silver saccharate.

#### Paper Chromatography of the Sugar Solution.

Paper : Whatman No.1.  $12\frac{1}{2}$ " x  $7\frac{1}{2}$ " cut along direction of machining. Method : Upward development for 11 hours<sup>(83)</sup>.

Solvent : n-butanol-ethanol-water (4-1-5),<sup>(84)</sup> the aqueous layer being removed and placed in a vessel at the bottom of the chromatography tank.

The sugar solutions (0.002ml.) were applied by means of a microsyringe to give round, compact spots (2 mm. diameter) at least one inch apart, in a line along the bottom of the paper (which had been previously equilibrated with the atmosphere in the developing tank prior to application of the sugars). After development, the papers were dried in air, and then sprayed with a mixture of 9 volumes of 0.2% solution of naphthoresorcinol in n-butanol and 1 volume of 89% phosphoric acid, the solutions being mixed immediately before use. The papers, after spraying, were dried in an oven at 100-105°, for 15 minutes, and the position and  $R_F$  values of the spots noted immediately, as the paper soon rots. A series of experiments were carried out, the time of development being increased to 12 and 14 hours respectively. Two spots were obtained in every case, one, designated spot (a), was small, round and compact, and the other, designated spot (b), was much larger, irregular and showed a good deal of tailing. These results are shown in Table 10.

Time (hours)	Spot	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	Average R <sub>F</sub>
11	(a)	0.453	0.442	0.432	0.404	0.394	0.425
	(b)	0.186	0.167	0.190	0.194	0.230	0.1934
12	(a)	0.457	0.457	0.430	0.416	0.400	0.433
	(b)	0.196	0.193	0.189	0.198	0.194	0.194
14	(a)	0.445	0.440	0.440	0.440	-	0.441
	(b)	0.200	0.196	0.194	0.199	-	0.1972

Table 10. Paper chromatography of the sugar solution for  
11, 12 and 14 hours.

In order to separate spot (b), the time was increased to 20 and 24 hours respectively, and the spray reagent now used was a 3% solution of p-anisidine hydrochloride in n-butanol, the paper being heated, after spraying, for exactly 10 minutes at 100°. Three distinct spots were obtained, designated (a), (b) and (c). The sugar solution was chromatographed along with a solution of leontosaponin, and 1% solutions of known sugars in water. These results are shown in Table 11.

Paper chromatography of the salt-free sugar solution and solutions, (1%), of glucose, and arabinose, are shown in Table 12.

Time (hours)	Solution	Spot and colour on spraying	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	Aver- age R <sub>F</sub>
20	Unknown sugar	(a) Yellow	0.426	0.430	0.430	0.434	0.430
		(b) Cherry-red	0.256	0.266	0.252	0.260	0.259
		(c) Yellowish- brown	0.219	0.216	0.214	0.215	0.216
	Leonto- saponin	Yellow	0.430	0.431	0.434	0.433	0.432
24	Unknown sugar	(a) Yellow	0.436	0.430	0.436	0.434	0.434
		(b) Cherry-red	0.262	0.254	0.267	-	0.261
		(c) Yellowish- brown	0.217	0.229	0.205	-	0.217
	Leonto- saponin	Yellow	0.434	0.434	-	-	0.434
	L-Arabinose	Cherry-red	0.266	0.260	0.268	-	0.265
	Fructose	Yellowish- brown	0.210	0.203	-	-	0.206
	Galactose	"	0.185	0.161	0.199	0.195	0.185
	D-Glucose	"	0.215	0.210	0.216	0.212	0.213
	Lactose	"	0.083	0.077	-	-	0.080
	Maltose	"	0.145	0.093	-	-	0.119
	Rhamnose	"	0.390	0.407	-	-	0.398
	Xylose	Cherry-red	0.284	0.296	-	-	0.290

Table 11. Paper chromatography for 20, and 24 hours.

Time (hours)	Solution	Colour of spot on spraying	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	R <sub>F</sub>	Average R <sub>F</sub>
	Unknown sugar	Cherry-red	0.268	0.260	0.258	0.269	0.263
		Yellowish- brown	0.214	0.224	0.218	0.216	0.218
24	L-Arabinose	Cherry-red	0.260	0.262	0.261	0.264	0.262
	D-Glucose	Yellowish- brown	0.226	0.224	0.215	0.215	0.220

Table 12. Paper chromatography of the salt free sugar solution, and solutions of D-glucose and L-arabinose, for 24 hrs.

Method of desalting the sugar solution<sup>(86)</sup>.

A mixture of activated charcoal and cellulose powder, (Whatman, standard grade), (1-1, 30g.) was packed in a column (27 x 3cm.). The cellulose had been previously washed with water to remove reducing substances, and dried, and the column was now washed with 150ml. of water. The sugar solution was concentrated to give an approximately 10% solution which was chromatographed on the column, eluting with water. 10ml. fractions of the eluate were collected and tested for the presence of a reducing sugar (Benedict's reagent) and pentose sugar, (aniline test) and the rotation noted. The

first 8 fractions contained no sugar, but contained salt. The next 24 fractions contained traces of salt, and gave reactions for reducing, and pentose, sugars. The last 5 fractions contained only traces of a reducing sugar, and no pentose sugar. All were dextro-rotatory. Fractions 9 to 32 and 33 to 36 were bulked and evaporated to dryness under reduced pressure at a temperature not exceeding  $60^{\circ}$ , to give a yellow sticky solid, which gave reactions for both reducing and pentose sugars, and also contained traces of salt. No more sugar could be eluted from the column, even with aqueous alcohol, 5% and 15%, respectively.

Separation of Arabinose and Glucose<sup>(87)</sup>. The crude sugar residue obtained (3.5g.), was dissolved in aqueous ethanol (50%, 14ml.), and mixed with an ethanolic solution of N-N-benzylphenylhydrazine (4.09g. in 33ml. ethanol). The solution on standing for 30 hours in a sealed vessel deposited arabinose N-N-benzylphenylhydrazone, which was recrystallised from 75% ethanol as colourless stout rods (2.18g.), m.p.  $173.5-174^{\circ}$  (decomp.), identical in every respect (melting point, mixed melting point) with genuine arabinose-N-N-benzylphenylhydrazone. (Found : C, 65.6; H, 6.8; N, 8.5. Calculated for  $C_{18}H_{22}O_4N_2$  : C, 65.4; H, 6.7; N, 8.5 per cent) The original mother liquors from the above salt were refluxed with a solution of formaldehyde (40%, 10ml.) for 1 hour on a

boiling water-bath, and the oily formaldehyde hydrazone extracted with ethyl acetate (8 x 25ml.). The aqueous solution gave the reactions of a reducing sugar, but did not give Bial's or the Aniline test. The solution was evaporated to dryness under reduced pressure, and the residue redissolved in water (1ml.), was refluxed on a water-bath with an ethanolic solution of diphenylhydrazine (0.66g; 17ml.). A portion of the ethanol (12ml.) was removed, and the solution treated with ether until there was a faint opalescence, the solution filtered rapidly whilst still hot, and left in a sealed vessel for 18 hours at 5°. The solid which separated was recrystallised from ethanol (charcoal) to give a crystalline solid (2.55g.), m.p. 159-160.5° (decomp.) identical in every respect with a genuine sample of glucose diphenylhydrazone. (Found : C, 62.6; H, 6.6; N, 8.1. Calculated for  $C_{18}H_{22}O_5N_2$  : C, 62.4; H, 6.4; N, 8.1 per cent.).

#### Isolation and characterisation of the sugars.

Isolation of L-arabinose. The N-N-benzylphenylhydrazone obtained above, was dissolved in aqueous ethanol (50%, 25ml.) and refluxed with a solution of formaldehyde (10ml.) for 1 hour as before. The oily formaldehyde hydrazone was extracted with ethyl acetate and the sugar solution evaporated to dryness to give a sticky, off-white solid which was recrystallised

from boiling ethanol (50ml.) and dried at  $100^{\circ}$ , gave L-arabinose as a white crystalline solid, m.p.  $159.5^{\circ}$ ,  $[\alpha]_D^{20} +104.5^{\circ}$  (c, 1.0 in water). It gave the reactions of a reducing, and a pentose sugar, and was identical (melting point, mixed melting point, and rotation) with a genuine sample of L-arabinose (m.p.  $160^{\circ}$ ,  $[\alpha]_D^{20} +105.4^{\circ}$  (c, 1.0 in water)).

Isolation of D-glucose  $\beta$ -pentaacetate<sup>(159)</sup>. Glucose diphenylhydrazone obtained above, was decomposed with formaldehyde, and the free sugar isolated as before. The sugar residue (2g.) and anhydrous sodium acetate (1g.) were refluxed with acetic anhydride (20ml.) on a water-bath until a clear solution was obtained (45 minutes), and thereafter for a further 2 hours. The reaction mixture was poured into ice-water (50ml.), and the sticky pale yellow solid which separated, was recrystallised from methanol as colourless needles, m.p.  $131^{\circ}$ ,  $[\alpha]_D^{20} +3.9^{\circ}$  (c, 1.0 in methanol), identical in every respect with genuine D-glucose  $\beta$ -pentaacetate.



CONSTITUTION OF LEONTOSAPONIN

Determination of the ratio glucose/arabinose using the method of Hirst and Jones (88). Leontosaponin (10mg.) and dilute sulphuric acid (0.5ml.) were heated together in a sealed tube, (10 x 0.5 cm., blown out to a bulb at one end), for 8 hours on a boiling water-bath. The tube was cooled, and the solution centrifuged, the genin collecting in the bulb. The tube was opened, and the solution carefully neutralised with solid barium carbonate, adding the latter carefully on the end of a glass-rod to avoid frothing of the solution. The solution was again centrifuged, and the sugar solution siphoned off. The residue was washed with water (0.2ml.), the latter removed after centrifugation, and the two solutions mixed to give 0.7ml. of a pale yellow sugar solution which contained approximately 7mg. of mixed sugars (calculated as glucose.). The solution (0.002ml.) was then spotted on to paper (Whatman No.1, 51 x 9 cm.) as shown in Fig. 8 and chromatograms developed by the method of downward development with the solvent system n-butanol-ethanol-water (4-1-5), for periods varying from 26-56 hours (after 50 hours the solvent ran off the paper).

The spots were applied carefully so that 1 cm. separated the spots, and three spots, two at each end, and one in the centre, were separated by 1" from the other spots to serve as

controls for obtaining the positions of the sugars after development. The two end and centre strips containing these

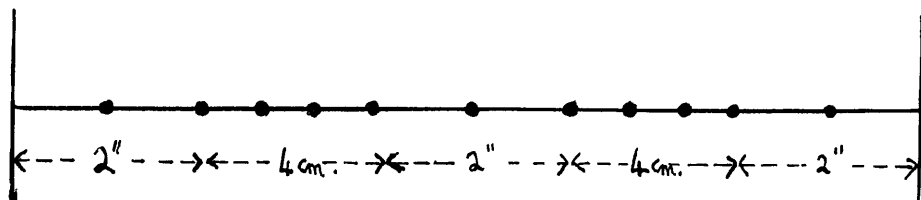


Fig.8. Method of applying spots to paper.

spots were removed after development, and the positions of the sugars determined by spraying with p-anisidine hydrochloride solution as before. By replacing these strips in the paper it was possible to cut four areas from each chromatogram, two containing only glucose, and the other two arabinose. The spots in all cases were separated from each other by distances varying from 0.3cm. to 1.0cm. depending on the time of the chromatogram. Areas, free of sugar, were also cut from the same paper as controls. The sugars were removed from the papers by suspending them from the end of a fine glass-rod, the end of which was hooked on to the lower joint of a spiral glass condenser. A boiling tube was attached to the lower joint of the condenser, 5ml. of water added, and refluxed for 30 minutes. Water condensed on the condenser, fell off the glass-rod, flushing the paper strips. After 30 minutes, no sugar could be detected on the paper. Meanwhile a 0.25M. solution of sodium metaperiodate in water

was prepared, which contained no free nitric acid. The sugar solutions obtained were oxidised with the metaperiodate solution (1ml.) for exactly 20 minutes on a boiling-water-bath, ethylene glycol, neutral to methyl red, (0.2ml.) added to the cooled solution to destroy excess periodate, and the formic acid produced titrated with N/100 sodium hydroxide solution to methyl red. A blank was done on the sugar free paper strips. At the end point the colour of the sugar solution was identical with the colour of the blank. The results are shown in Table 13.

Time (hours)	24	24	36	40	40	50	50	56	56	Average ratio
Amount of sugar (mixture) mg.	0.48	0.62	0.75	0.38	0.44	0.51	0.61	0.59	1.07	-
Ratio:										
glucose	$\frac{4}{2.51}$	$\frac{4}{2.85}$	$\frac{4}{3.28}$	$\frac{4}{2.82}$	$\frac{4}{2.98}$	$\frac{4}{3.22}$	$\frac{4}{3.11}$	$\frac{4}{3.01}$	$\frac{4}{3.06}$	$\frac{4}{3}$
arabinose										

Table 13. Determination of the ratio glucose/arabinose.

Determination of moisture in leontosaponin<sup>(28)</sup>. Leontosaponin, (0.1498g.) was dried in an oven at 120° to constant weight. Loss 5.58%. In two repeats, (0.1006g.) and (0.25g.) leontosaponin lost 5.63% and 5.67% respectively. Average loss

5.62%. Calculated for  $C_{69}H_{112}O_{36} \cdot 5H_2O$  : 5.604% .

Enzymatic hydrolysis of leontosaponin.

(A) Leontosaponin (0.5g.) in distilled water (100ml.) gave a solution of pH 6.6. 5ml. of this solution was mixed well with 1ml. of a standard solution of  $\beta$ -glucosidase (1%), the time noted, and the solutions placed in a constant temperature bath at  $40^{\circ}$ . At 10 minute intervals for the first 2 hours, and  $\frac{1}{2}$  hour intervals thereafter, 2 drops of the solution were removed and tested with Benedict's reagent to give the results shown in Table 4. At the same time a second 5ml. of saponin solution was treated with a 1% solution of the enzyme material isolated from L. leontopetalum, whilst a further 5ml. was used as a control for the above two tests, the solutions being tested as before. The results are shown in Table 4 (page 44a).

(B) Method of Sumner and Howell<sup>(89)</sup>. Leontosaponin (0.572g) was dissolved in 100ml. of acetate buffer, (pH 4.4), prepared by mixing N/10 acetic acid (630ml.) with N/10 sodium acetate solution (370ml.). 5ml. portions of this solution were treated with the standard solutions of  $\beta$ -glucosidase, and enzyme solution, 5ml. being again used as a control. The solutions, mixed well, were put in a bath at  $40^{\circ}$  as before to give the results shown in Table 5 (page 44a).

ISOLATION AND CHARACTERISATION OF  
LEONTICINE, LEONTAMINE AND PETALINE

Extraction of the drug by Method 1.

(a) Water. The aqueous liquid left after the isolation of crude saponin, fraction A (page 16), was acidified with dilute sulphuric acid, filtered, basified with ammonia, and extracted with chloroform (10 x 25ml.). The combined chloroform extracts were washed with water, dried (sodium sulphate), and evaporated to give a dark-brown, gummy alkaloid fraction, A, (0.03g. 0.3%). Ether did not extract any further alkaloid from the mother-liquors, which still gave alkaloidal reactions. The alkaline solution was therefore acidified to pH 4.0 with dilute sulphuric acid, and excess Mayer's reagent added, to give a brown amorphous solid, fraction B (0.042g. 0.42% after drying in vacuo).

The marc left after extraction with boiling water, was macerated for 36 hours with a 1% solution of hydrochloric acid (100ml.), and chloroform-soluble, and water-soluble alkaloids isolated as before to give 0.025g., and 0.012g., of fractions A, and B, respectively.

(b) Cold ethanol. The drug (50g.) was macerated with ethanol for 24 hours in a sealed vessel, then percolated to

exhaustion with the same solvent (4.5l.). The filtrate was concentrated to a soft extract, and saponin removed as before (Method 1, page 23). The alcohol-ether solution was concentrated to dryness, and the residue dissolved in dilute sulphuric acid, filtered, and separated as before into a chloroform-soluble alkaloid, fraction C, and a water-soluble alkaloid, isolated as the Mayer complex, fraction D, the yields being 0.28g., (0.56%), and 0.32g., (0.64%) respectively. The marc, on extraction with 1% acid as before gave a further 0.12g. (0.24%) of fraction C, and 0.055g., (0.11%) of fraction D.

Hot ethanol. Extraction of the drug with hot ethanol as described in Methods (1), (2) and (3), (pp.23 and 24), for the isolation of the saponin, gave (after isolation of saponin) alkaloidal residues which were bulked, and separated as before into a dark-brown sticky chloroform-soluble fraction, 9.92g. (0.57%) which was added to fraction C, and a dark-brown Mayer-complex of the water-soluble alkaloid, 12.9g. (0.75%) which was added to fraction D. The marcs from Methods (1), (2) and (3), on percolation with acid as before gave further yields of fraction C, 1.88g., (0.11%), and 0.8g., (0.046%) of fraction D.

Extraction of the drug by Method II.

The drug (100g.) was completely exhausted by maceration (24 hours) and subsequent percolation with 1% hydrochloric acid (1830ml.). A portion of this percolate (610ml.) was made alkaline with ammonia, and completely extracted with chloroform (15 x 25ml.) as before, to give a dark-brown, gummy alkaloid fraction, E (0.213g., 0.64%). The water-soluble alkaloid was precipitated as the Mayer complex, as before, to give fraction F, (0.24g., 0.72%), a dark-brown amorphous solid which did not melt below  $360^{\circ}$ .

A further portion of the percolate (310ml.) was treated with excess (50ml.) of a solution of lead subacetate (10%), and the voluminous, buff precipitate obtained filtered, and washed with dilute acid. The pale yellow filtrate and washings were extracted as before, to yield chloroform-soluble alkaloid, (0.082g., 0.48%) fraction G, and a pale-brown amorphous Mayer-complex, fraction H, (0.073g., 0.43%).

A further portion of the filtrate (310ml.) was heated on a boiling water-bath for 30 minutes with activated charcoal (2.5g.), and the charcoal washed with dilute acid. The pale yellow filtrate and washings were extracted as before to give 0.019g., (0.14%) of chloroform-soluble alkaloid, which was added to fraction G, and 0.017g., (0.1%) of a pale-brown Mayer-complex, which was added to fraction H. The lead

subacetate and charcoal residues were dried, and refluxed with ethanol for 4 hours, when the lead subacetate gave 0.022g., and the charcoal 0.0391g., of total alkaloid. No further yields of alkaloid could be recovered from the residues.

The remainder of the acid filtrate (610ml.) was extracted with ether (20 x 50ml.). Very persistent emulsions were formed which were not easily broken with alcohol, stirring, heat (50°), or by the addition of salt. The ether extracts were united, washed with water, dried (sodium sulphate), and the solvent removed to give a dark-brown, non-alkaloidal residue. Extraction with chloroform (20 x 50ml.) also caused emulsification, and again the emulsions were very stable. Evaporation of the chloroform extracts gave a dark-brown, sticky, non-alkaloidal solid. The acid solution was then treated with Mayer's reagent (30ml.), and the total alkaloid-Mayer complex isolated as a dark-brown, amorphous solid (0.614g., 1.84%).

The crude alkaloid fractions obtained in Methods I and II.

(a) The chloroform-soluble alkaloid. Fractions A, C and E, were bulked to give 12.25g. of a dark-brown sticky solid which gave reactions for alkaloid, saponin, and reducing sugar. It could not be obtained as a powderable solid on drying in vacuo, nor crystallised from ethanol, methanol,



chloroform, benzene, aqueous ethanols (60-90%), or mixtures of ethanol-ether, or ethanol-benzene. The fraction (12g.) was treated with dilute hydrochloric acid when 2.9g. of dark-brown saponin was obtained. The acid solution, basified with ammonia, was re-extracted with chloroform, the above process repeated (twice) to give 7.85g. of a dark-brown, sticky solid, which still contained traces of saponin, and which could not be crystallised, nor dried in vacuo to a powderable solid. The fraction (0.5g.) in ethanol (4ml.) with ethanolic picric acid (5%, 4ml.), gave a dark yellow, hygroscopic picrate, which could not be crystallised nor purified.

The fraction (0.5g.) in ethanol (4ml.), with excess (7ml.) of a saturated ethanolic hydrogen chloride solution, gave a brown gummy hydrochloride, which could not be crystallised, nor purified, m.p. 150-180° (decomp.).

The alkaloid fraction (6.8g.) in dilute hydrochloric acid (200ml.), was basified with caustic soda (20%), and extracted with chloroform (21 x 30ml.) to give 5.9g., of a dark-brown, gummy alkaloidal fraction, I.

The mother-liquors, treated with excess saturated aqueous ammonium chloride, and extracted with chloroform (20 x 20ml.) gave 0.6g. of a dark-brown, semi-solid alkaloid fraction, J, which again could not be dried in vacuo to a powderable solid.

The mother-liquors, which contained only traces of water-soluble alkaloid, not extracted by either chloroform, or ether, were not investigated further.

Fraction I. 0.5g. portions were treated with ethanolic picric acid, and ethanolic hydrochloric acid, solutions, as before, to give a gummy impure picrate and hydrochloride respectively, which failed to crystallise.

Fraction J was insoluble in caustic soda solution; it gave no colour with ferric chloride, did not give the Liebermann-nitroso reaction, and failed to couple with p-nitrobenzene-diazonium chloride. Treatment of this fraction (0.3g.) with water, left only a small insoluble portion (0.09g.), which was dissolved in dilute acid, basified with sodium hydroxide solution, and extracted with chloroform to give a dark-brown, gummy alkaloid (0.06g.), which was combined with fraction I. Treatment of the mother-liquors with ammonium chloride, and extraction with chloroform gave a dark, gummy alkaloid, fraction J<sub>(1)</sub>, (0.02g.) which was reserved for chromatographic examination, along with 0.1g. of the original fraction J, (see page 169).

Fraction G. 0.05g. portions were treated with ethanolic picric acid, and ethanolic hydrochloric acid, as before, to give a gummy, impure picrate and hydrochloride, which could

not be crystallised.

(b) Water-soluble alkaloid fractions.

The Mayer-complexes, fractions B, D and F, were bulked (14.23g.), and the complex decomposed by the following methods:-

- (i) The complex (5g.) was shaken continuously for 1 hour with a saturated sodium carbonate solution (30ml.), the solution filtered, and carefully neutralised with acid. The solution was evaporated to dryness, and the residue extracted with hot ethanol, filtered, and the filtrate evaporated to give a dark-brown, semi-solid alkaloid (1.81g., 36.2% of complex).
- (ii) The complex (5g.) was heated on a water-bath with a saturated solution of sodium carbonate (30ml.), for 30 minutes, filtered, neutralised and the water-soluble alkaloid isolated as (i) above, to give a dark-brown, semi-solid alkaloid (2.1g., 42%).
- (iii) The complex (4.23g.) was shaken with sodium hydroxide solution (20%) for 1 hour at 40°, filtered, and the water-soluble alkaloid isolated as in (i) above, to yield 1.98g. (42.7%) of a dark-brown, sticky alkaloid.

The alkaloid residues so obtained were almost completely

soluble in water and ethanol, slightly soluble in ether and chloroform, but insoluble in benzene, carbon tetrachloride, and caustic alkalis. They could not be crystallised from ethanol, methanol, or mixtures of ethanol-ether, or ethanol-benzene. 0.5g. Fractions of the crude alkaloid in ethanol were treated with ethanolic picric acid, and ethanolic hydrochlorid acid, as before, when in both cases gummy, impure products were obtained, which were hygroscopic, and could not be crystallised.

Fraction H. 0.09g. was decomposed as in method (iii) above to give a pale-brown, gummy alkaloid fraction (0.038g., 42%) which again could not be crystallised. The residue when treated with ethanolic picric acid, again gave a gummy hygroscopic picrate which failed to crystallise.

The total alkaloid-Mayer complex (0.61g.) (page 131) was decomposed as in method (iii) above. The filtrate was neutralised with hydrochloric acid, basified with ammonia and extracted with chloroform (20 x 10ml.), to give 45.8mg. of a pale-brown, gummy alkaloid residue which again could not be crystallised and gave a gummy picrate. The mother-liquors evaporated to dryness under reduced pressure and the residue extracted with ethanol, gave, on removal of the ethanol, a dark-brown, sticky alkaloid (0.21g.) which was insoluble in chloroform, but soluble in water. It could not be

crystallised and again gave a gummy picrate.

Extraction of the drug by Method III.

(a) The drug (50g.) was moistened with ammonia for 12 hours in a sealed vessel, and the damp drug extracted in a Soxhlet apparatus with dry benzene (300 ml.) for 16 hours on a water-bath. The benzene was washed with water, dried (sodium sulphate), and the solvent removed to give a dark-brown, semi-solid alkaloid fraction (0.21g., 0.42%). This fraction was insoluble in water, and did not give reactions for saponin. The marc left after extraction with benzene, was dried and extracted with boiling ethanol to give a dark-brown, semi-solid extract. This was dissolved in acid, filtered, made alkaline with ammonia, and extracted with chloroform to give a further small yield (0.025g., 0.05%) of alkaloid. The filtrate was acidified to pH 4, and treated with excess saturated aqueous phosphotungstic acid, when a slimy greenish-brown precipitate of the water-soluble alkaloid complex was obtained. The precipitate, which was filtered with difficulty, was washed with water, and dried in vacuo to give a dark greenish-brown amorphous solid, fraction K (0.389g., 0.75%) which did not melt below  $360^{\circ}$ .

(b) The drug (200g.) was mixed with powdered calcium hydroxide (60g.), and moistened with water for 12 hours.

The damp mass was sifted through a No.10 sieve, divided into three portions (i), (ii) and (iii), and treated as follows:-

- (i) was macerated with 70% ethanol (24 hours), then exhausted by percolation with 70% ethanol (3.5l.).
- (ii) was macerated with 90% ethanol (24 hours), then exhausted by percolation with 90% ethanol (2.75l.).
- (iii) was macerated with absolute ethanol (24 hours), then exhausted by percolation with absolute ethanol (2.52l.).

The filtrate obtained in each case was concentrated in vacuo. On cooling the concentrate, saponin was precipitated and removed. The remainder of the saponin was removed by precipitation with ether and the saponin washed thoroughly with the minimum amount of dry ethanol. The filtrates, and washings, were evaporated to dryness under reduced pressure. The residues, dissolved in dilute hydrochloric acid, were filtered, basified with ammonia, and extracted with chloroform to give dark-brown, semi-solid alkaloid fractions, which also gave reactions for saponin, and reducing sugars. Yields:-

(i) 0.42g., (0.63%); (ii) 0.44g., (0.65%), and (iii) 0.48g., (0.72%). These fractions were bulked to give fraction L. The mother-liquors, acidified with hydrochloric acid to pH 2.0, and treated with saturated aqueous mercuric chloride, yielded a precipitate of the water-soluble base as

the mercuric-chloride complex, a dark-brown, amorphous solid which did not melt below  $360^{\circ}$ . Yields:-

(i) 0.5g., (0.74%); (ii) 0.53g., (0.8%); and (iii) 0.54g., (0.81%). These fractions were bulked to give fraction M. The marcs from (i) and (ii), percolated with 1% hydrochloric acid, yielded a small amount of alkaloids; the marc from (iii) similarly treated gave only traces of alkaloids.

(c) The drug (5Kg.) was moistened for 12 hours in a sealed vessel with dilute solution of ammonia, sieved, and packed into a tin-lined copper percolator. The drug was macerated with ethanol for 24 hours, and percolated to exhaustion with ethanol (10.5l.). The percolate, concentrated to 3l., was cooled, and filtered from precipitated saponin, which was washed with ethanol. The filtrate and washings were evaporated to dryness and the residue dissolved in dilute sulphuric acid, filtered, made alkaline with ammonia, and extracted with chloroform (22 x 50ml.). The combined chloroform extracts were washed with water, dried (sodium sulphate), and evaporated to give a dark-brown, gummy alkaloid (22.85g., 0.46%), which was added to fraction L. The ammoniacal mother liquors (1l.) were acidified to pH 2.0 with dilute sulphuric acid, and the water-soluble alkaloids precipitated as the mercuric chloride complex as before, to give a brown amorphous solid, (39.1g., 0.78%), which was added to fraction M.

The crude alkaloid fractions obtained by Method III.

Attempts to remove saponin from fraction I.

(i) Fraction I, (6.43g.), was dissolved in dilute hydrochloric acid (25ml.), filtered from insoluble non-alkaloidal solid, (0.73g.), and the solution passed through an activated column of Zeo-Karb 225 (30g., 13 x 1cm.). The column was washed free of acid, and the alkaloid eluted with concentrated hydrochloric acid (65ml.). The eluate was diluted with water, basified with ammonia, and extracted with chloroform to yield a dark-brown, semi-solid alkaloid (4.79g., 74.5% recovery) which was free of saponin. Ammoniacal ethanol failed to elute further amounts of alkaloid from the column.

(ii) Fraction I, (1g.), was dissolved in dilute hydrochloric acid (10ml.), filtered from insoluble material, and poured on to a column (30g., 13 x 1cm.) of activated I.R.C.50 resin. About 0.65g. of alkaloid (65%) passed through the column without being adsorbed. The remainder was eluted as in (i) to give 0.1g. (10%), of a dark-brown, semi-solid alkaloid, which was free of saponin.

(iii) Fraction I, (5g.), was refluxed with dry benzene (25ml.) on a water-bath for 4 hours, and the benzene filtered from undissolved material, (saponin, and a trace of water-soluble alkaloid), which was washed with benzene. The benzene



solution was evaporated to give a dark-brown, sticky alkaloid (3.4g., 68% recovery), which did not contain saponin, nor reducing sugar. The product was completely soluble in ethanol and chloroform, and insoluble in water. Dilute hydrochloric acid removed a small insoluble portion (0.26g.), which was non-alkaloidal.

The remainder of fraction L, (11.8g.), was refluxed with benzene, filtered, and the benzene evaporated to give a saponin-free chloroform-soluble alkaloid (7.13g.).

Preparation of a reineckate from the benzene-soluble alkaloid.

The crude alkaloid (54mg.) was suspended in water (3ml.), and treated with excess (8ml.) of saturated aqueous ammonium reineckate solution, when a pale pink amorphous reineckate was obtained, m.p. 180-182° (decomp.), after careful recrystallisation from 60% aqueous acetone at 50°. The reineckate (50mg.) in acetone (10ml.) was spotted on to paper (Whatman No.1) by means of a microsyringe (0.005ml., 25 µg. of reineckate), and chromatographed by upward development for 16 hours, using the solvent system pyridine-water (1-4). After drying, the papers were sprayed with a solution of potassium bismuth iodide in dilute acetic acid<sup>(102)</sup>, when reineckate spots were obtained as orange-pink spots on a yellow background, the colours fading on standing. Three spots were obtained,  $R_F$  values 0.92, 0.62 and 0.50. The first was a brownish-red,

faint, small, round spot; the second a pink, more distinct, small, round compact spot; the third was orange-pink, large and irregular, with a good deal of tailing.

Chromatography of the benzene solution of the alkaloid on alumina (103).

The alkaloid fraction (10g.) in dry benzene (50ml.) was chromatographed on a column of alumina (200g., 25 x 3cm.), which had been previously washed with dry benzene (350ml.). The column was eluted with benzene (1.5l.), to give 7.65g. (76.5% recovery) of a dark-brown, semi-solid base. Further elution with benzene containing 1% ethanol (1l.) gave a further 2.22g., 22.7% of a dark-brown, semi-solid base. Reineckates were prepared from each of these fractions (0.1g.) designated (i) and (ii) respectively, and purified by chromatography in acetone solution (on alumina (5g., 3 x 0.5cm) and elution with acetone (50ml.). Concentration of the eluates, precipitation of the reineckates with water, and careful recrystallisation of the products from aqueous acetone as before, gave the reineckates as pink, micro-crystalline solids, both having m.p. 219-221° (decomp.). Paper chromatography of reineckates (i) and (ii) as before, showed both to be a mixture of two reineckates,  $R_F$  0.441, a large, irregular tailing spot, and  $R_F$  0.723, a small, round compact spot.

The benzene-soluble alkaloid still could not be dried in vacuo to give a powderable solid, and this fraction, (10g.), was dissolved in dilute hydrochloric acid (200ml.), basified with ammonia, and extracted first with ether (25 x 30ml.), and then with chloroform (18 x 25ml.).

The ether-soluble alkaloid fraction, obtained as a pale-brown oil (3.25g.), on treatment with warm ethanol (80%) (charcoal) yielded a colourless crystalline alkaloid (0.288g.), m.p. 118.5-119.5°,  $[\alpha]_D^{20} \pm 0^\circ$  (c, 1.0). The crystals gave reactions with Mayer's and Dragendorff's reagents, iodine potassium iodide, picric acid, and phosphotungstic acid solutions. The bulk of the fraction (2.8g., 86.7%) could not be crystallised. The total ether-soluble alkaloid fraction gave a pink, microcrystalline reineckate m.p. 218-221° (decomp.) which was prepared, and chromatographed on paper as before, when two spots were obtained, a small, round compact spot  $R_F$  0.725, and a large, irregular, tailing spot  $R_F$  0.465.

The chloroform-soluble alkaloid fraction was obtained as a dark-brown, semi-solid alkaloid fraction 4.2g., from which nothing crystallised, and which could not be dried in vacuo to a powderable solid. The product (0.5g.) with ethanolic picric acid gave a brown, gummy picrate, m.p. 130-140° (decomp.), which was hygroscopic and could not be crystallised. Similar treatment of the base (0.5g.) with ethanolic hydro-

chloric acid gave an impure, deliquescent hydrochloride, of indeterminate melting point.

The base (0.5g.) in warm ether (50ml.) was treated with an ethereal solution of anhydrous oxalic acid<sup>(160)</sup>. A sticky brown precipitate was obtained which was washed with ether, but failed to recrystallise from alcohol-acetone.

A portion of the base (1.0g.) was distilled in vacuo, to give a yellow oily alkaloid (0.3g.) b.p. 150-160° (bath)/4mm.Hg.

The reineckate, prepared as already described, was obtained as a pinkish-red, crystalline solid, m.p. 219-222° (decomp.) which when chromatographed on paper gave two spots, a very small, round, compact spot  $R_F$  0.72, and a large irregular, tailing spot  $R_F$  0.444.

(b) Water-soluble alkaloid complexes.

(i) The Phosphotungstic acid complex (0.37g.) was suspended in barium hydroxide solution (50ml.) and heated on a water-bath for 30 minutes. Excess baryta was precipitated with solid carbon dioxide, the precipitate filtered, washed with water, and the filtrate and washings evaporated to dryness under reduced pressure, when the water-soluble alkaloid was obtained as a dark-brown, semi-solid (0.17g., 44.5% of complex). It could not be crystallised, nor completely dried

to a powderable solid in vacuo. The alkaloid (0.2g.) in ethanol (2ml.) with excess of ethanolic picric acid, gave a dark yellow picrate which was hygroscopic, and failed to crystallise, m.p. 130-150° (decomp.). The alkaloid (0.2g.) in ethanol (2ml.), with excess ethanolic hydrochloric acid gave a gummy hygroscopic hydrochloride, the melting point of which could not be accurately determined.

(ii) The Mercuric chloride complex (40.67g.) was dissolved in ethanol (95%, 200ml.), and hydrogen sulphide passed in until the solution assumed a uniform black colour. It was filtered, the residue washed free of alkaloid with water, and the filtrate and washings evaporated to dryness under reduced pressure, when the water-soluble alkaloid chloride was obtained as a dark-brown amorphous, deliquescent solid (19.85g., 48.8% of complex). The product was dissolved in water, filtered, and the filtrate decolourised with activated charcoal (3g.) to give a yellow solution. Further treatment with charcoal removed most of the base, but not the yellow colour. The filtrate was evaporated to give a yellow amorphous deliquescent solid (12.5g., 0.25% of the root-tubers), m.p. 130-140°. It could not be crystallised.

Extraction of the drug with boiling ethanol.

The drug (30g.) was extracted in a Soxhlet with ethanol

(100ml.) for 48 hours, the ethanol being replaced every 12 hours with fresh solvent. The ethanol extracts were united and the saponin removed in the usual way. The filtrate was concentrated to dryness, the residue dissolved in dilute acid, filtered, and reserved for future examination. The marc was extracted by percolation with dilute acid, but no alkaloid was obtained.

LARGE SCALE EXTRACTION OF ALKALOIDS AND SAPONIN

(a) The mother-liquors remaining after the extraction of the saponin from the 1.9Kg. of crude extract obtained on alcohol extraction (pages 101-102), were acidified with dilute sulphuric acid, filtered, and combined with the acid filtrate obtained in the last experiment, basified with ammonia, and extracted with chloroform (17 x 50ml.) to give 36.4g., 0.64% of a crude dark-brown, chloroform-soluble alkaloid fraction. The ammoniacal solution remaining after the isolation of chloroform-soluble alkaloids was acidified with dilute sulphuric acid to Congo-red, and treated with saturated aqueous ammonium reineckate solution (9l.) to yield the crude water-soluble alkaloid reineckate (55.94g.).

(b) The remaining 3.5Kg. of crude extract (pages 101-102) was dissolved in dilute sulphuric acid (5l.) and treated similarly, to give a further 66.5g., 0.727% of crude chloroform-soluble alkaloid which was added to that previously obtained, and a further 216g. of crude brownish-red, reineckate which was added to that previously obtained.

Purification of the chloroform-soluble alkaloids. All the crude chloroform-soluble alkaloid fractions previously obtained were added to the fractions obtained above, and this fraction (109.) mixed with powdered cellulose (100g.), and

continuously extracted in a Soxhlet with dry benzene (500ml.) for 24 hours. The benzene solution was removed, washed with water, dried (sodium sulphate), and evaporated to give a dark-brown, sticky alkaloidal residue, 64g. This was dissolved in dilute sulphuric acid, and was extracted first with ether (26 x 50ml.), and then with chloroform (20 x 50ml.). Both these extracts were washed with water, dried (sodium sulphate), and evaporated to give 9.92g. and 14.95g. of dark-brown, non-alkaloidal residues, which were discarded. The acid solution was basified with ammonia, and extracted with ether (24 x 50ml.), and then chloroform (18 x 50ml.):—

The ethereal extracts were bulked, washed with water, dried (sodium sulphate), and evaporated to give a pale-brown viscid oil (11.6g.). This partly crystallised from 80% methanol as small colourless glistening needles of leonticine, m.p. 118.5 - 119.5° (0.975g., 0.007% of the drug). The oily, non-crystallisable alkaloid residue (10g.), was distilled in vacuo to give a pale yellow oil (5.6g.), b.p. 150-160° (bath)/4mm.Hg.,  $n_d^{20}$  1.5411, which after repeated distillation gave leontamine as a colourless, mobile, oily base, b.p. 155-160° (bath)/4mm.Hg.,  $n_d^{20}$  1.5115 (3.44g., 0.024% of the drug). The non-distillable residue (4.3g.), a dark-brown, semi-solid, gave alkaloidal reactions and this fraction, N, was reserved for chromatographic examination (page 169).



The chloroform extracts, similarly gave 22.7g. of a brown, semi-solid alkaloid which crystallised from methanol (80%) to give leonticine as a colourless crystalline alkaloid (1.6g.), m.p. 118.5-119.5° (undepressed on admixture with that obtained above),  $[\alpha]_D^{20} \pm 0^\circ$  (c, 1.0). The oily, non-crystallisable residue (19.9g.), on repeated distillation, gave leontamine as a colourless oily alkaloid, b.p. 140-145°/7mm.Hg., 118-120°/4mm.Hg.,  $n_D^{17} 1.5120$ ,  $d_{20}^{20} 0.986$  (8.1g., 0.06% of drug) identical with that obtained above.

Leonticine was obtained as small colourless needles, m.p. 118.5-119.5°,  $[\alpha]_D^{20} \pm 0^\circ$  (c, 1.05),  $\epsilon$ , 25,957 at 218 m $\mu$  and  $\epsilon$ , 25,538 at 296 m $\mu$ . (Found: C, 73.3; H, 7.6; N, 4.4.  $C_{20}H_{25}O_3N$  requires : C, 73.3; H, 7.7; N, 4.3 per cent.). It was insoluble in water, slowly soluble in ether, more readily so in chloroform, and very soluble in ethanol or methanol.

Leonticine Hydrochloride. Leonticine (0.8g.) in ethanol (5ml.) was treated with ethanolic hydrochloric acid, and the solution evaporated to dryness under reduced pressure. Recrystallisation from ethanol-ether gave leonticine hydrochloride as pale yellow, deliquescent needles, m.p. 125-126° (decomp.).

Leonticine Picrate. Leonticine (0.4g.) in ethanol (2ml.), treated with excess of ethanolic picric acid, gave leonticine picrate as glistening yellow needles, m.p. 172-173° (decomp.) (from ethanol). (Found : C, 56.01; H, 4.84; N, 10.07.  $C_{26}H_{28}O_{10}N_4$  requires : C, 56.1; H, 5.07; N, 10.06 per cent.).

Leonticine Reineckate. Leonticine (0.2g.) in ethanol (5ml.) treated with excess saturated aqueous ammonium reineckate gave leonticine reineckate as dark-red microprisms (from aqueous acetone), m.p. 166-167° (decomp.) with preliminary darkening at

163°. Mixed melting point with leontamine reineckate (m.p. 225-226° (decomp.)), 183-185° (decomp.). Paper chromatography of this reineckate, as before, showed it to be homogeneous, with  $R_F$  0.73.

Leonticine Chloroplatinate. Leonticine hydrochloride (0.2g.) in water (4ml.) was treated with aqueous chloroplatinic acid (5ml., 5%) containing a few drops of dilute hydrochloric acid, and the orange-yellow amorphous solid recrystallised (water) as bright yellow needles, m.p. 162-163° (decomp.). (Found : C, 32.19; H, 3.6; Pt, 26.2;  $C_{20}H_{25}O_3NH_2PtCl_6$  requires : C, 32.57; H, 3.69; Pt, 26.47 per cent.).

Attempted hydrogenation of leonticine.

Leonticine (0.2g.) in ethanol (5ml.) and platinum oxide (0.03g.) was shaken with hydrogen for 6 hours. No significant uptake of hydrogen occurred. Filtration and evaporation of the ethanol gave leonticine, m.p. 118-119°.

Leontamine was obtained as a colourless, mobile, oily liquid b.p.  $118-120^{\circ}/4\text{mm.Hg.}$ ;  $100-102^{\circ}/2\text{mm.Hg.}$ ;  $140-145^{\circ}/7\text{mm.Hg.}$ ;  $n_{\text{d}}^{20} 1.5117$ ,  $d_{20}^{20} 0.787$ ,  $[\alpha]_{\text{D}}^{20} + 2.78$  (c, 1.061). (Found : C, 75.4; H, 11.4; N, 12.5. Calculated for  $\text{C}_{14}\text{H}_{26}\text{N}_2$  : C, 75.6; H, 11.8; N, 12.6 per cent.). Leontamine was insoluble in water, sparingly soluble in ether, more readily soluble in chloroform, and readily soluble in ethanol and methanol. The ultra-violet absorption spectrum showed no absorption.

Application of Hinsberg separation to leontamine.

Leontamine (1g.), sodium hydroxide solution (20ml., 10%), and benzene sulphonylchloride (3ml.) were mixed, warmed on a water-bath for 30 minutes, and the alkaline solution acidified with dilute hydrochloric acid. No precipitate was obtained (absence of primary and secondary bases), and the solution was basified with sodium hydroxide, and extracted with chloroform (10 x 15ml.) as before to give leontamine (0.88g.),  $n_{\text{d}}^{20} 1.5115$ .

Attempted hydrogenation of leontamine. Leontamine (0.5g.) in ethanol (10ml.) and platinum oxide (0.05g.), was shaken with hydrogen for 8 hours. No significant uptake of hydrogen occurred and the solution was filtered, and evaporated to give leontamine (0.48g.),  $n_{\text{d}}^{20} 1.5118$ .

Test for unsaturation. Leontamine (2mg.) in ethanol (0.05ml.) gave no yellow colour (30 minutes) with a solution of tetranitromethane in carbon tetrachloride.

Leontamine Hydrochloride. Leontamine (1g.) in dry ethanol (10ml.) was treated with sufficient ethanolic hydrochloric acid to make the solution just acid. Addition of dry acetone to the warm solution until turbid gave, on cooling, leontamine hydrochloride in two crystalline forms, mainly long slender needles, m.p.194.5-197°, and a small amount of minute cubic crystals, m.p.188-190°, which were partly separated by hand picking. Mixed m.p. of (a) and (b), 192-196°. Yunusov and Sorokina<sup>(31)</sup> give for leontamine hydrochloride m.p.192-196°. Further treatment with ethanolic hydrochloride, and recrystallisation from ethanol-acetone, again gave two crystal forms, mainly as long slender needles with a surface covering of minute prisms, m.p.192-196°.

The mixture of hydrochlorides was very soluble in water, methanol and ethanol, but was insoluble in acetone, chloroform, benzene and ether. It could not be resolved.

Leontamine Picrate. The hydrochloride (0.3g.) in water(4ml.) was treated with aqueous sodium picrate solution (saturated, 6ml.) to give leontamine picrate, m.p.193.5-195° (decomp.) from ethanol. (Found : C, 48.01; H, 6.2. Calculated for

$C_{26}H_{32}N_8O_{14}$  : C, 48.29; H, 6.6 per cent.). It was insoluble in water, sparingly soluble in ethanol, readily soluble in hot ethanol, and in ethyl acetate and acetone.

Leontamine Chloroplatinate. The hydrochloride (0.3g.) in water (4ml.) was treated with 5% aqueous chloroplatinic acid (8ml.) containing a few drops of dilute hydrochloric acid. The orange-yellow, amorphous precipitate was recrystallised from boiling water containing a little hydrochloric acid, to give leontamine chloroplatinate as shining orange needles, m.p. 248-248.5° (decomp.) with preliminary darkening at 244-245°. (Found : C, 26.9; H, 4.5;  $PtO_2$ , 34.4. Calculated for  $C_{14}H_{26}N_2H_2PtCl_6$  : C, 26.6; H, 4.43;  $PtO_2$ , 35.9 per cent).

Leontamine Dimethiodide. Leontamine (1g.) in methanol (7ml.) was refluxed on a boiling water-bath for 7 hours with methyl iodide (2ml.). The methanol was removed and the residue recrystallised from boiling ethanol to give leontamine dimethiodide as a white microcrystalline solid, m.p. 265-266° (decomp.). (Found : C, 37.85; H, 6.2; N, 5.9. Calculated for  $C_{14}H_{26}N_2(CH_3I)_2$  : C, 37.9; H, 6.3; N, 5.6 per cent.). The dimethiodide was readily soluble in water, to give a neutral solution, but was sparingly soluble in ethanol.

Leontamine Reineckate. Leontamine hydrochloride (0.5g.) in

water (15ml.) was treated with excess of saturated aqueous ammonium reineckate, and the amorphous solid recrystallised from aqueous acetone (60%, 50°) to give leontamine reineckate as pink glistening platelets, m.p. 225-226° (decomp.) with preliminary darkening at 222°. Paper chromatography of this reineckate as before showed leontamine to be homogeneous, giving a spot,  $R_F$  0.43.

THE WATER-SOLUBLE ALKALOID, PETALINE

The crude reineckate (272g.) was treated with dry acetone (2l.) and filtered from insoluble matter (non-alkaloidal) (88.7g.). The acetone filtrate and washings (3.2l.) were concentrated (1 in 5) in vacuo, and chromatographed on alumina (1.6Kg.). Elution with acetone gave three fractions, of which the first, and the last, were yellow, and contained neither alkaloid nor saponin. They fluoresced a bright yellow in U.V. light and on evaporation to dryness gave only a trace of a yellow solid, which did not melt below  $360^{\circ}$ , and was not examined further. The middle fraction was concentrated to small volume (50ml.) below  $50^{\circ}$ , and the reineckate precipitated by addition of water (150ml.). Recrystallisation from aqueous acetone (60%,  $50^{\circ}$ ) gave petaline reineckate (100g., 36.8% of the crude reineckate) as a pink microcrystalline solid, m.p.  $179-181^{\circ}$  (decomp.). Paper chromatography of this reineckate as before, showed it to be homogeneous,  $R_F$  0.68. (Found : C, 44.77; H, 5.2 . Equiv. wt. 678.2, 679.5.  $C_{20}H_{22}O_3N \cdot [Cr(SCN)_4(NH_3)_2]$  requires : C, 44.8; H, 4.4. Equiv. wt. 642.8).

Decomposition of Petaline Reineckate. The reineckate (100g.) in acetone (2l.) was titrated with silver sulphate solution (0.6%) until no further precipitate was obtained. The



equivalent of barium chloride was added, the solution filtered, and the precipitates washed with aqueous acetone (20%). The combined filtrate and washings, evaporated under reduced pressure at a temperature not exceeding  $50^{\circ}$ , gave 54.9g. of a greenish-yellow scale-like solid, which could be powdered to a greenish-yellow deliquescent solid. The product gave reactions with the usual alkaloidal reagents, and was free from saponin. It was dissolved in dry methanol, filtered from insoluble material (5.5g.), and the solution evaporated to dryness. The solid was treated with water, filtered from insoluble chromic sulphate (12.7g.). Repetition of this process several times removed all the chromic sulphate (3.0g.), and the filtrate on evaporation gave petaline chloride as a deep yellow, scale-like deliquescent solid, (33.6g., 0.24% of the root-tubers). Petaline chloride, dried in vacuo had m.p.  $140-143^{\circ}$  (decomp.),  $[\alpha]_D^{20} +11.3$  (c, 1.0 in water)  $\epsilon$ , 20,576, at  $224\text{ m}\mu$ , and  $\epsilon$ , 11,600, at  $280\text{ m}\mu$  and  $\epsilon$ , 334 at  $328\text{ m}\mu$ . (Found : C, 63.35; H, 6.8; N, 3.8; OMe 16.9.  $\text{C}_{18}\text{H}_{16}\text{O}(\text{OCH}_3)_2\text{N}\cdot\text{Cl}\cdot\text{H}_2\text{O}$  requires : C, 63.4; H, 6.7; N, 3.9; OMe 17.2 per cent.). It is readily soluble in cold and hot water, cold and hot ethanol or methanol, acetic acid, and butanol, only slightly soluble in chloroform, and less so in ether. It is completely insoluble in benzene, light petroleum, and carbon tetrachloride. Petaline chloride could not be crystallised from

dry ethanol, methanol, ethanol-ether, or ethanol-acetone. The aqueous solution gave no colour with ferric chloride solution, but an ethanolic solution gave a very faint green colour. An aqueous solution of petaline chloride (1%), mixed with a solution of p-nitrobenzenediazonium chloride gave an oily orange-brown solid, which was not a typical phenol reaction. Petaline chloride did not give the Liebermann-nitroso reaction for phenol. Petaline chloride gave the following reactions:-

- (1) Bromine water - a yellow flocculent precipitate which turned orange on washing with sulphurous acid.
- (2) Concentrated sulphuric acid - a yellow colour, turning olive green on warming.
- (3) Concentrated nitric acid - a brownish-red colour.
- (4) Chlorine water - a brownish-red solution.
- (5) Labat test<sup>(108)</sup>. An alcoholic solution of petaline chloride (0.1ml., 10%), gallic acid (5%, 0.1ml.) and concentrated sulphuric acid (2ml.) were heated on a water-bath. No green nor blue colour was obtained.
- (6) Froehde's reagent - a bottle-green colour.

Petaline Picrate. Petaline chloride (0.48g.) in water (5ml.) was treated with saturated aqueous sodium picrate to give petaline picrate, as elongated microprisms from absolute ethanol, m.p. 165.5-166° (decomp.). (Found : C, 56.43; H, 4.4; OMe, 11.39; N, 10.09.  $C_{26}H_{24}O_{10}N_4$  requires :

C, 56.52; H, 4.4; OMe, 11.2; N, 10.1 per cent.)

Petaline Chloroplatinate. Petaline chloride (100mg.) in water (10ml.) acidified with 2 drops of dilute hydrochloric acid, was treated with a 5% aqueous platinic chloride solution to give petaline chloroplatinate, as a yellow amorphous solid, which was recrystallised from ethanol as a pale yellow microcrystalline solid, m.p. 197-198° (decomp.).

(Found : C, 44.34; H, 4.1; Pt, 18.18.  $(C_{20}H_{22}O_3NCl)_2PtCl_4$  requires : C, 45.46; H, 4.4; Pt, 18.47 per cent.).

Petaline Sulphate. Petaline chloride (0.5g.) in water (10ml.) was treated with one equivalent of silver sulphate, the precipitated silver chloride filtered, washed with water, and the combined filtrate and washings evaporated to yield petaline sulphate as a scale-like, dark yellow, deliquescent solid. It was dried in vacuo, m.p. 88-89° (decomp.).

Petaline Nitrate. Petaline chloride (0.4g.) in water (10ml.) was treated with the calculated quantity of silver nitrate solution, the precipitated silver chloride filtered, washed with water, and the filtrate and washings evaporated to give petaline nitrate as a dark yellow, scale-like, very deliquescent solid, m.p. could not be determined.

Petaline Perchlorate. Petaline chloride (0.65g.) was slowly added to perchloric acid (5ml., 75%) and water (5ml.). A yellow solid, petaline perchlorate, was obtained, which was recrystallised from hot ethanol as yellow needles, which were hygroscopic, m.p. 114-115° (decomp.) with preliminary fusion at 88-90° (in a sealed tube).

Petaline Iodide. Petaline chloride (1g.) in water (20ml.) was treated with excess of a 10% aqueous solution of potassium iodide, to give a yellow sticky solid, recrystallised from ethanol-ether (4-1) as yellow prisms. It darkened on heating at 190°, fused at 230° and began to char, but did not melt.

Attempted preparation of Petaline Acetate.

Petaline chloride (0.513g.), anhydrous sodium acetate (0.12g.), and acetic anhydride (2ml.) were refluxed at 120° for 2 hours, after solution had occurred ( $\frac{1}{2}$  hour.). The dark-brown solution was poured into ice-water (10ml.). No precipitate was formed; the solution was neutralised with sodium carbonate solution, and extracted with ether. The ether was washed with water, dried (sodium sulphate), and evaporated to give only a trace (1.2mg.) of a dark-brown solid, which did not give the reactions of an acetate. The aqueous mother-liquors were acidified to Congo-red, and the

base precipitated as the reineckate in the usual manner. The reineckate was purified, as before, to give a pink microcrystalline reineckate, (0.87g.), m.p.178-180° (decomp.), undepressed on admixture with petaline reineckate. The reineckate was decomposed as before to give a yellow scale-like solid, which was dissolved in ethanol, filtered, and evaporated to give petaline chloride, as a yellow, deliquescent solid, m.p.141-143°, undepressed on admixture with genuine petaline chloride. The ultra-violet absorption spectrum of the base chloride obtained, confirmed its identity as petaline chloride.

## HYDROGENATION OF PETALINE CHLORIDE

### Dihydropetaline Chloride.

Petaline chloride (0.4g.) in acetic acid (5ml., 50%) was hydrogenated in the presence of Adam's catalyst (0.0444g.). Absorption was complete in 9 hours, at 17°. (Found : 24.83ml. absorbed at N.T.P. Calculated for one double bond  $C_{20}H_{24}O_3NCl \cdot 2H_2O$  requires : 25.17ml. at N.T.P.). The solution was filtered, and evaporated to dryness under reduced pressure to give dihydropetaline chloride as a colourless, hygroscopic solid, m.p. 122-125° (decomp.),  $[\alpha]_D^{20} -16.7^\circ$  (c, 0.2 in water). ( $\epsilon$ , 15,364) at 224 m $\mu$  and ( $\epsilon$ , 11,580) at 280 m $\mu$ . The solid slowly became pale yellow on standing. (Found : C, 60.3; H, 6.91; O, 20.13; N, 3.6; OMe, 15.4.  $C_{20}H_{24}O_3NCl \cdot 2H_2O$  requires : C, 60.36; H, 7.09; O, 20.1; N, 3.5; OMe, 15.6 per cent.)

### Dihydropetaline Picrate.

Dihydropetaline chloride (0.2g.) in water (5ml.) was treated with excess of saturated aqueous sodium picrate, and the amorphous product, recrystallised from aqueous ethanol (80%), gave dihydropetaline picrate as yellow needles, m.p. 161.5-162.5° (decomp.). Mixed m.p. with petaline picrate, 164.5-165° (decomp.). (Found : C, 56.06; H, 4.6; O, 28.8; N, 10.0.  $C_{26}H_{26}O_{10}N_4$  requires : C, 56.3; H, 4.72;

O, 28.85; N, 10.1 per cent.)

### Dihydropetaline Chloroplatinate.

Dihydropetaline chloride (0.0765g.) in water (2ml.) was treated with an aqueous solution of platinic chloride (2ml., 5%), and the buff coloured precipitate recrystallised from water as yellow needles, m.p. 179-180° (decomp.) with preliminary darkening at 143°. Mixed m.p. with petaline chloroplatinate, 189-192° (decomp.). (Found : C, 45.12; H, 4.7; Pt, 18.3.  $(C_{20}H_{24}O_3NCl)_2PtCl$  requires : C, 45.28; H, 4.56; Pt, 18.40 per cent.)

### Reduction of Petaline Chloride with Zinc.

Petaline chloride (1.27g.), zinc dust (2g.) and dilute hydrochloric acid (10ml.) were refluxed, when the yellow solution gradually became colourless (5-10 minutes). After 30 minutes the solution was cooled, filtered, and the zinc washed with dilute acid until free of adsorbed alkaloid. The filtrate and washings were basified with excess ammonia, and the ammoniacal solution first extracted with ether, and then with chloroform. Ether and chloroform extracted only insignificant amounts of base. The ammoniacal solution was acidified to Congo-red, and the base precipitated as the reineckate which was purified, and decomposed as before to give a colourless, hygroscopic solid, m.p. 123-126° (decomp.).

undepressed on admixture with dihydropetaline chloride. The ultra-violet absorption spectrum of the base chloride confirmed this identification as dihydropetaline chloride, and this was also verified by preparation of a picrate and chloroplatinate, identical with those of dihydropetaline.



ACTION OF ALKALI ON PETALINE CHLORIDE

(a) Petaline chloride (0.5g.) in ethanol (5ml.) was exactly neutralised with alcoholic sodium hydroxide, the precipitated sodium chloride filtered, washed with ethanol, and the filtrate and washings evaporated to dryness. A black resinous solid was obtained, which could not be induced to crystallise. It was only slightly soluble in ether or chloroform, but readily soluble in water, and ethanol. It could not be distilled in vacuo.

(b) Petaline chloride (0.5g.) in water (5ml.) was treated with a slight excess of an aqueous solution of sodium hydroxide (20%). Extraction of this solution with ether (10 x 10ml.) and chloroform (10 x 10ml.) did not extract any alkaloid. The aqueous solution, on evaporation gave a black residue which again could not be crystallised.

(c) Petaline chloride (0.5g.) in water (5ml.) was exactly neutralised with a 4% aqueous solution of barium hydroxide. Extraction of this solution with ether and chloroform did not extract any alkaloid, and evaporation of the solution gave a black resinous solid which failed to crystallise.

(d) Petaline chloride (0.5g.) in water (5ml.) was treated with excess of a 4% aqueous solution of barium hydroxide. The solution on standing at room temperature for 8-12 days yielded 15-20% of the base as a chloroform-soluble base. The base (0.101g.) was obtained as a dark-brown, semi-solid which gave the usual alkaloidal reactions. Recrystallisation from aqueous ethanol (80%) (charcoal) gave a trace (15mg.) of pale-brown needles, m.p. 117-118°.

(e) Petaline chloride (2.5g.) in water (20ml.) was treated with excess of a 4% aqueous solution of barium hydroxide, and the solution evaporated to dryness. The almost black residue was extracted with chloroform (5 x 100ml.). The chloroform extracts were united, washed with water, dried (sodium sulphate), and evaporated to give a dark brown, semi-solid (0.75g., 30% of petaline chloride), which was recrystallised from 80% ethanol (charcoal) as almost colourless needles, m.p. 117.5-118.5°, undepressed on admixture with leonticine. Ultra-violet absorption spectra on the base confirmed this identification, and further confirmation was shown by the preparation of a picrate, m.p. 172.5-173.5° (decomp.), and a chloroplatinate, m.p. 162-163.5° (decomp.), both melting points undepressed on admixture with leonticine picrate and chloroplatinate. The base also gave a reineckate, m.p. 166-167° (decomp.) which when chromatographed on

paper as before, gave one spot,  $R_F$  0.71. (cf. leonticine reineckate  $R_F$  0.73.)

The chloroform-insoluble residue was dissolved in dilute hydrochloric acid and the base precipitated as a reineckate which was purified as before to give a pale pink, micro-crystalline solid, m.p. 162-164° (decomp.). Paper chromatography of this reineckate showed it to have an  $R_F$  0.60. It was decomposed, as before, to give a pale yellow hygroscopic solid, the alkaloid chloride, m.p. 105-110° (decomp.), the melting point being very difficult to determine owing to the facility with which the substance absorbed moisture. Unlike petaline chloride, the solid did not give satisfactory analytical results, a series of microanalyses showing widely different results, and the solid now gave the reactions of a ketone. It has therefore been designated oxypetaline chloride.

Oxypetaline 2:4-dinitrophenylhydrazone.

Oxypetaline chloride (0.1g.) in water, was made distinctly acid with sulphuric acid and treated with a solution of 2:4-dinitrophenylhydrazine in aqueous sulphuric acid to give a dull orange-red amorphous solid, which recrystallised from aqueous alcohol (80%) as micro-cubes, m.p. 143-144° (decomp.). Again a series of microanalyses did not give consistent results. An empirical formula has therefore not been assigned to oxypetaline.

OXIDATION OF PETALINE CHLORIDEAlkaline permanganate oxidation (119)

Petaline chloride (1.12g.) in water (50ml.) at 90° was slowly mixed with a solution of potassium permanganate (1.8g.) and potassium carbonate (0.3g.) in water (100ml.) at 90°, stirring during the reaction. After the addition of all the permanganate solution (20 minutes) the product was cooled under the tap and the precipitated manganese dioxide filtered, and washed with water. The pale-brown filtrate and washings were treated with sulphur dioxide (passed in a slow stream through the solution) to give a brownish-yellow amorphous solid which was filtered off, washed with water and dried in vacuo, fraction (A), (0.186g.).

The manganese dioxide precipitate was suspended in water, and re-dissolved by passing a slow stream of sulphur dioxide through the mixture. A brownish-yellow liquid was obtained which contained in suspension a brown amorphous solid, which was filtered, washed with water, dried in vacuo (0.001g.), and added to fraction (A).

Fraction (A), m.p. 130-220° (decomp.), was suspended in water, and shaken at 40° with a slight excess of sodium carbonate solution (10%) to give (i) a soluble portion (0.108g.) and (ii) an insoluble portion (0.079g.).

(i) The soluble portion in the filtrate was treated with excess dilute sulphuric acid and the dark-brown sticky solid obtained, m.p. 196-236° (decomp.), filtered, washed, dried and recrystallised from methanol (charcoal) as a colourless, amorphous, non-nitrogenous acid, m.p. 194-206° (decomp.) (0.014g.). The charcoal was re-extracted with methanol to give only a further 0.004 g. of solid. It was not further investigated.

(ii) The insoluble portion, a pale-brown sticky solid, m.p. 110-130° (decomp.) gave the reactions of an alkaloid. It was dissolved in boiling 90% acetic acid (2ml.); on cooling, a sticky solid was obtained. This solid could not be crystallised from ethanol or methanol, m.p. 110-130° (decomp.). Insufficient material was obtained to permit further investigation.

The original mother-liquors from the oxidation were concentrated in vacuo to small volume, filtered whilst hot from a large amount of salt. The filtrate was then concentrated to dryness and the almost black sticky residue extracted with boiling ethanol, and methanol. These solvents did not remove significant amounts of material. No further product could be isolated from the above residue.

Paper chromatography of fractions J and J(1).

The crude alkaloid fractions were treated with ammonium reineckate solution and the crude reineckates purified and chromatographed on paper as before.

Fraction J was found to consist of a mixture of three alkaloids,  $R_F$ 's 0.73, 0.60 and 0.43 respectively (for the reineckates). The majority of the fraction was that of  $R_F$  0.60 (cf. 0.68 for petaline reineckate), thus verifying the theory that the majority of this so-called phenolic alkaloid was the water-soluble alkaloid, petaline. The  $R_F$  values of the other two alkaloids show them to be leonticine and leontamine respectively, verifying the previous findings that only three alkaloids are present in the plant.

Fraction J(1) similarly consisted of the same three alkaloids, although as most of the water-soluble alkaloid had been removed from this fraction, the majority of this fraction was leontamine ( $R_F$  0.44) with only traces of petaline and leonticine.

Paper chromatography of fraction N

Fraction N was treated as above, and the reineckate chromatographed on paper as above, when it was found to be homogeneous,  $R_F$  0.45, although there was slight tailing of the spot, due to impurities. Fraction N was very impure,

and consisted of decomposed leontamine. No other alkaloid was found in this fraction.

# EXAMINATION OF THE LIGHT PETROLEUM SOLUBLE EXTRACT

The dark-brown semi-solid petrol extract (31.1g.) was melted in a flask with water (500ml.), and steam distilled for 4 hours. The distillate (3.5l.) was extracted with light petroleum (30 x 50ml.), the petrol bulked, washed with water, dried (sodium sulphate), and the solvent removed under reduced pressure to give a pale yellow essential oil (0.94g.) which still contained a trace of solvent. The oil was kept in an oven at 60° for 12 hours, and then in a vacuum desiccator over phosphorus pentoxide for 2 days, when 0.53g. of a pale yellow oil was obtained which had a characteristic pleasant odour,  $[\alpha]_D^{20} +4.15^\circ$  (c, 1.09),  $n_D^{17} 1.3706$ ,  $d_{17}^{17} 0.830$ .

The oil did not deposit any crystalline material on keeping at 0°. It did not give a Schiff's reaction for aldehyde, but gave a yellow precipitate with ethanolic 2:4-dinitrophenylhydrazine, insufficient in quantity to isolate a crystalline product. The oil (5 drops) in petrol (2ml.), rapidly decolourised a solution of bromine in petrol (2ml., 1.0%). Evaporation of the solvent gave an almost colourless oil, which was only with difficulty crystallised to give oily colourless needles, m.p. 67-69°. Insufficient material was obtained to permit its characterisation.



Examination of the non-volatile material. The non-volatile fatty residue was repeatedly extracted with petrol (5 x 50ml.), the petrol bulked, washed with water, dried (sodium sulphate), and the solvent removed to give a dark-brown, semi-solid (29.9g.).

Saponification of the non-volatile material<sup>(124)</sup>. The fat (29.9g.) was refluxed with ethanolic potassium hydroxide (60g. of potassium hydroxide in 300ml. of 70% ethanol) on a boiling water-bath until solution occurred (1hour), and thereafter for a further 3 hours. The ethanol was removed, water being added to keep the volume constant, and the soap solution cooled, diluted with water (90ml.), and extracted with ether (1 x 240ml., followed by 4 x 100ml.). The ether solutions were bulked, washed with water (3 x 50ml.), the washings being added to the soap solution. The ether was dried (sodium sulphate), and the solvent removed to give 5.6g. of a yellow viscid oil which contained traces of crystalline material. This residue was again refluxed with ethanolic potassium hydroxide (10ml.) for 10 minutes on a boiling water-bath, and re-extracted as before to give 5.58g. of a yellow viscous oil, fraction I, containing traces of crystalline material.

Acidification of the aqueous liquid with hydrochloric acid, precipitated the fatty acids as an almost colourless

solid. This solid was extracted with light petroleum, (3 x 300ml.), the petrol washed with water, dried (sodium sulphate), and evaporated to give the fatty acid (11.2g.), fraction II. The aqueous liquid still contained a little oily material which floated on the surface of the water, and this was extracted with ether (3 x 300ml.) to give an oily solid, 2.37g. which could not be induced to crystallise, and was not further investigated.

The aqueous acid liquor constituted fraction III, a pale yellow liquid.

FRACTION I.      UNSAAPONIFIABLE MATTER.

This fraction was dissolved in petrol (150ml.) and chromatographed on a column of alumina (310g., 48 x 3cm.) which had been previously washed with petrol. Elution with petrol and the appropriate solvents gave the results recorded in Table 6 (page 76).

Fraction A. A colourless oil (0.57g.) deposited crystals on cooling, which were collected and recrystallised from ethyl acetate as soft lustrous plates, which coalesced on filtering, giving a wax-like cake, m.p. 56-61°. Recrystallisation from ethanol gave soft, pearly plates (21.4mg.) m.p. 63-64°, which did not coalesce on filtering. (Found : C, 84.89; H, 14.4. Calculated for  $C_{31}H_{64}$  : C, 85.23; H, 14.8 per cent.). The product was neutral in reaction, insoluble in water, but soluble in hot ethanol, less readily soluble in cold ethanol, and very soluble in petrol, and benzene. The solid (4mg.) in petrol (1ml.) with bromine in petrol (1ml., 1%) caused no decolourisation, and similarly the solid (4mg.) in carbon tetrachloride (1ml.) and solution of tetranitromethane (1ml.) caused no yellow colouration.

The remainder of fraction A, a colourless oil, could not be crystallised, and attempted distillation under reduced pressure gave no volatile material. This portion of the fraction was also saturated.

Fraction B. A colourless oil (0.5g.) which on cooling deposited crystals, m.p. 57-70°. Recrystallisation from a large volume of ethanol, and rapid cooling, gave a small amount of pearly leaflets, m.p. 63-64°. Concentration of the mother liquors to dryness, and treatment of the residue with light petroleum gave a further yield of crystals, m.p. 63-64°. The petrol insoluble portion, recrystallised from ethyl acetate, gave ceryl alcohol, (0.029g.), as colourless crystals, m.p. 77-77.5°,  $[\alpha]_D^{20} \pm 0^\circ$  (c, 1.102) (Found : C, 81.3; H, 14.36. Calculated for  $C_{26}H_{54}O$  : C, 81.6; H, 14.2 per cent).

Ceryl Acetate. The above alcohol (0.015g.) was refluxed with acetic anhydride (2ml.) for 1 hour. Ethanol (2ml.) was added, the solution boiled for a further 30 minutes, cooled, and diluted with water. The precipitate was extracted with ether (5 x 20ml.), and the ether layer washed successively with brine (2 x 5ml.), saturated sodium bicarbonate solution (2 x 10ml.), water (3 x 5ml.), and dried (sodium sulphate). Evaporation of the ether, and recrystallisation of the residue from ethyl acetate, gave small leaf crystals of m.p. 60.5-62.5°. (Ceryl acetate m.p. 63°, 64°, 65° Beilstein<sup>(161)</sup>).

As before, most of fraction B could not be crystallised, nor could it be distilled in vacuo.

Fractions C, D and E. These were not examined.

Fraction F. A deep yellow oil (3.26g.) deposited crystals (1.86g.) on cooling, which were obtained as colourless rosettes of slender needles m.p.155.5-156.5° (from methanol), (1.41g.).

The sterol had an  $[\alpha]_D^{20} +4.30^\circ$  (c, 1.1 in benzene),  $[M]_D + 19.01$  ( $\Delta_1$ ).  $\xi$ , 8,058 at 208 m/ $\mu$ , and  $\xi$ , 775 at 280 m/ $\mu$  as shown in Fig.9. (Found : C, 81.7; H, 10.98.  $C_{29}H_{46}O.CH_3OH$  requires : C, 81.45; H, 11.39 per cent.).

The crystals gave the following colour reactions:-

- (1) Liebermann-Burchard reaction<sup>(133)(134)</sup>. A few crystals were dissolved in 2 drops of chloroform, treated with acetic anhydride (1ml.) and concentrated sulphuric acid (2 drops), to give the following colours:-  
mauve  $\longrightarrow$  blue  $\longrightarrow$  bottle green.
- (2) Salkowski reaction<sup>(135)</sup> gave a brown colour in the chloroform, and a blood-red colour in the acid layer.
- (3) Rosenheim reaction<sup>(162)</sup>. No yellow colour was obtained.
- (4) Mach's reaction<sup>(163)</sup>. A few crystals of sterol were evaporated to dryness with hydrochloric acid and 2 drops of ferric chloride solution. The residue, washed well with water, gave a bright red colour.
- (5) Moleschott's reaction<sup>(163)</sup>. A few crystals, moistened with a mixture of 1 part of water and 5 parts of

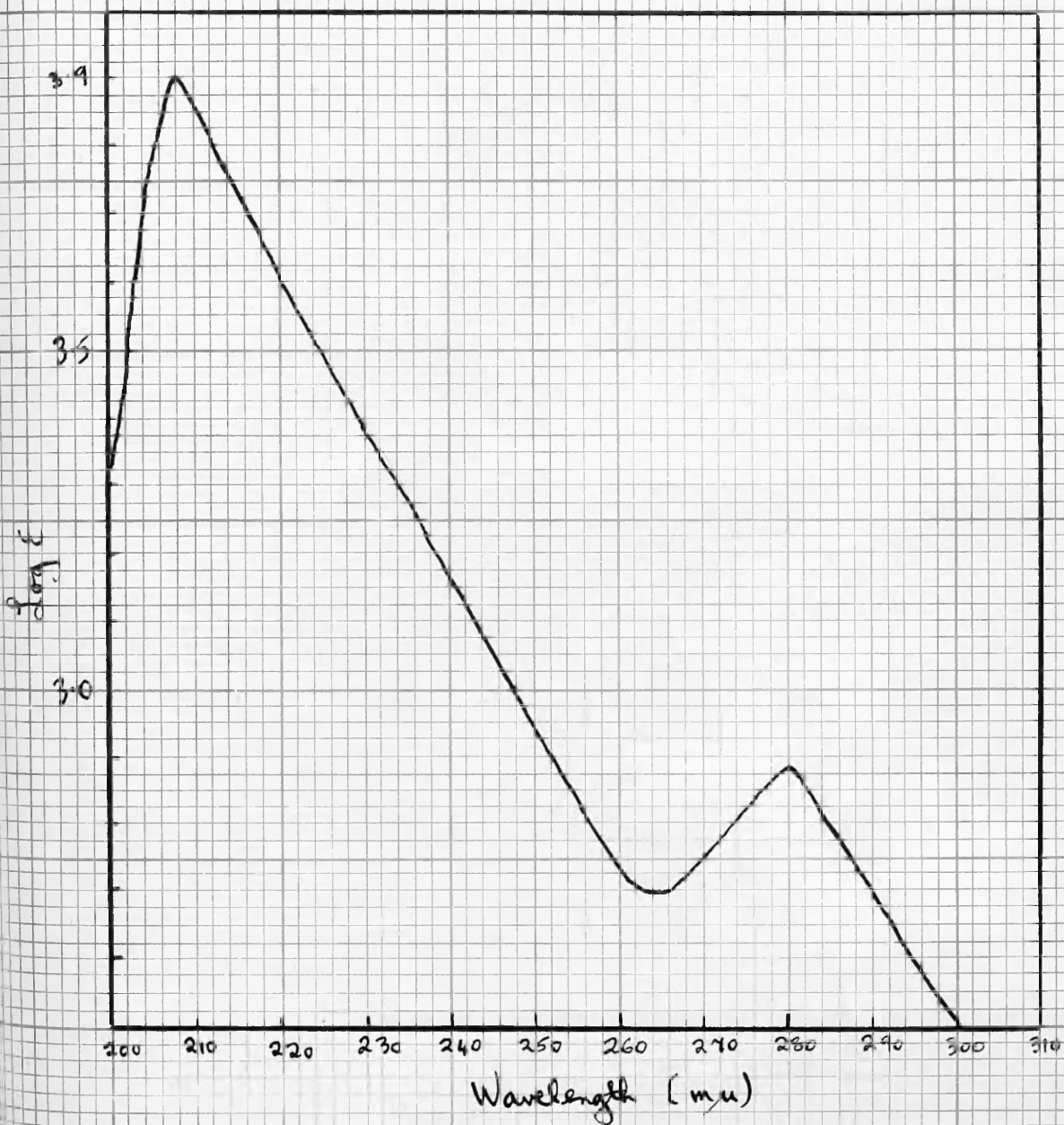


Figure 9. Ultra-violet absorption spectrum of the phytosterol.

sulphuric acid, were coloured red.

- (6) Tortelli-Jaffe' reaction<sup>(136)(137)</sup>. To a solution of the sterol in glacial acetic acid was added a 2% solution of bromine in chloroform, pipetting the latter carefully under the surface of the acid. A green ring appeared at the interface.

A solution of digitonin (0.5% in 95% ethanol) precipitated the sterol from ethanol solution<sup>(164)</sup> as a pearly, microcrystalline solid, which gave the Liebermann-Burchard reaction.

Hydrogenation of the phytosterol. The sterol (3.273mg.) in ethyl acetate (4ml.) was hydrogenated with a platinum catalyst. Uptake of hydrogen, 0.366ml. at N.T.P.

$C_{29}H_{46}O.CH_3OH$  requires for 2 double bonds, hydrogen uptake of 0.367ml. at N.T.P.

Phytosterol Acetate. The sterol (0.675g.) was heated with acetic anhydride (15ml.) for 4 hours on a boiling water-bath, the mixture diluted with ethanol (20ml.), and heated for a further 30 minutes under reflux. The solution was diluted with water, cooled, centrifuged, and the supernatant liquid rejected. The residue was recrystallised from ethanol as glistening plates, m.p. 159-160°,  $[\alpha]_D^{20} + 5.88^\circ$  (c, 0.17 in chloroform),  $[M]_D + 26.61$  ( $\Delta_2$ ). (Found : C, 82.32;

H, 10.9.  $C_{31}H_{48}O_2$  requires : C, 82.24; H, 10.7 per cent.).

Saponification of the sterol acetate. The acetate (0.45g.) was heated with ethanolic potassium hydroxide (5%, 15ml.) under reflux for 4 hours. Excess water was added, and the precipitated solid extracted with ether (3 x 50ml.). The ether extracts were united, washed with water, dried (sodium sulphate), and evaporated to dryness. The residue recrystallised from methanol as colourless rosettes of needles, m.p. 155.5–156.5°  $[\alpha]_D^{20} + 4.4^\circ$  (c, 1.0 in chloroform). (Found : C, 81.65; H, 11.1.  $C_{29}H_{46}O \cdot CH_3OH$  requires : C, 81.45; H, 11.39 per cent.).

Sterol Acetate Bromide. The acetate (0.2g.) in ether (3ml.), treated with a slight excess of a 5% solution of bromine in glacial acetic acid, gave a solution which slowly turned pale green, and did not deposit any solid material. The solution was treated with ethanol (5ml.), and water added to the warm (40°) solution until turbid. On cooling, a small amount (0.008g.) of a pale brown crystalline solid was obtained, which was recrystallised from aqueous ethanol as a pale yellow crystalline solid m.p. 228–230° (decomp.). The substance gave a precipitate of silver bromide with silver nitrate solution.



Phytosterol Benzoate. The sterol (0.5g.) was heated with pyridine (1.5ml.) and benzoyl chloride (0.03ml.), on a boiling water-bath for 1 hour. The cooled mixture was diluted with ether (25ml.), and the ether layer washed with dilute hydrochloric acid (2 x 25ml.), 2% sodium hydroxide (2 x 5ml.), and water (5 x 5ml.). The ether was dried (sodium sulphate), and the solvent removed to give a pale yellow solid which was recrystallised from benzene-ethanol (3-5) as beautiful lustrous plates, m.p. 179-180°  $[\alpha]_D^{20} +9.166^\circ$  (c, 0.12 in chloroform),  $[M]_D + 52.68$  ( $\Delta_3$ ) (Found; C, 83.79; H, 10.09.  $C_{36}H_{50}O_2$  requires : C, 83.99; H, 9.89 per cent.).

Molecular rotation differences of sterol and sterol acetate, and sterol and sterol benzoate.

$$\Delta_2 - \Delta_1 = + 7.6$$

$$\Delta_3 - \Delta_1 = + 33.67$$

For  $\Delta^7$  - stenols:-

$$\Delta_2 - \Delta_1 = - 30 \rightarrow \pm 0$$

$$\Delta_3 - \Delta_1 = + 34 \rightarrow + 6$$

Attempted ozonolysis of the phytosterol<sup>(165)</sup>

The sterol (0.1g.) in glacial acetic acid (10ml.) was ozonised for  $2\frac{1}{4}$  hours without cooling. The temperature

did not rise above room temperature, and the sterol did not dissolve. The solution, together with 5ml. of glacial acetic acid from rinsing the ozonolysis tube, was diluted with water (50ml.), and distilled through a preheated efficient fractionating column. The distillate (30ml.) gave no reactions for aldehyde. The sterol was recovered unchanged. A control experiment on ergosterol similarly gave no result, although an aldehyde should have been produced in this case. Lack of material, however, prevented further ozonolysis experiments.

Fraction G. This was recrystallised from methanol to give a further 0.1g. of phytosterol, m.p. 152-155°. The mother-liquors from fractions F and G, on evaporation, gave a yellow oil which gave reactions of a sterol, but which could not be crystallised and was not further investigated.

Fractions H and I. These could not be induced to crystallise, and were not further investigated.

FRACTION II      FATTY ACIDS

The acids (11g.) were dissolved in ethanol (95%, 50ml.), and the solution heated to boiling. A boiling solution of lead acetate (8g.) and glacial acetic acid (1ml.) in ethanol (50ml.) was added and the mixture cooled overnight. The pale yellow crystalline precipitate obtained was filtered, washed with 95% ethanol and recrystallised (twice), from 95% ethanol (50ml.) which contained glacial acetic acid (1ml.), as a pale yellow lead salt, m.p. 96-99°. The combined filtrate and washings were reserved for the examination of the unsaturated acids.

Decomposition of the lead salts of the saturated acids.

The salts were warmed with hydrochloric acid (20ml.) and water (20ml.), until a layer of fatty acids formed on the surface of the mixture, which was then cooled and transferred to a separator. The fatty acids were extracted with light petroleum (5 x 100ml.), and the bulked light petroleum layers washed free from lead salts and mineral acid with water. After drying the solution (sodium sulphate), the solvent was removed to give a pale yellow solid, (2.52g.), m.p. 44-47°.

Esterification of the saturated acids. The acids were dissolved in methanol (25ml.) which contained sulphuric acid (0.5ml.) and boiled gently under reflux for 2.5 hours. The

esters were isolated by diluting the solution with brine and extracting with light petroleum (2 x 150ml.), and the latter washed successively with brine, water, saturated solution of sodium bicarbonate, and water. The light petroleum layer was dried (sodium sulphate), and evaporated to give a pale yellow oil which solidified when cooled.

Fractional distillation of the methyl esters of the saturated acids. This was carried out using a short fractionating column. Fractions were collected at intervals, and the equivalent weight of ester (E.W.), and iodine value (I.V.) determined by the methods described below, on selected fractions. The results are summarised in Table 7 (page 84).

Method of determining the equivalent weight of the ester. The ester (0.5-1.0g.) accurately weighed, was dissolved in ethanol (5ml.) previously neutralised to phenolphthalein, and boiled gently under reflux with ethanolic potassium hydroxide solution (10ml., 0.6N.) for 1 hour. The mixture was cooled, and the excess of alkali titrated with 0.1N. hydrochloric acid using phenolphthalein as indicator (a ml.). A blank determination was carried out (b ml.). The saponification equivalent was calculated from the formula:-

$$\frac{\text{Weight of ester} \times 10,000}{(\underline{b} - \underline{a})}$$

Method of determining the iodine value. The ester (0.05 - 0.06g.) accurately weighed, was dissolved in carbon tetrachloride (5ml.) in a glass-stoppered flask, and a solution of iodine monochloride (British Pharmacopoea; 20ml.) added. After 30 minutes, potassium iodide (1g.) and water (50ml.) were added, and the liberated iodine titrated with 0.1N. sodium thiosulphate (a ml.). A blank determination was carried out (b ml.). The iodine value was calculated from the formula:-

$$\frac{(\underline{b} - \underline{a}) \times 0.01269 \times 100}{\text{weight of ester}}$$

Fraction (a). Identification of palmitic acid. Isolation of the acid from fraction (a) after its saponification gave a crystalline solid readily divided into two fractions by means of acetone. The acetone-insoluble fraction was crystallised from ethanol as a colourless, microcrystalline solid (0.296g.) m.p. 99-101°. This acid (0.2g.) was dissolved in ethanol and titrated against caustic soda solution to phenolphthalein. Equivalent weight, 351.5. The acid gave no reactions for lead. It was not examined further due to lack of material.

The acetone-soluble acid was precipitated by the addition of water to the acetone solution, and recrystallised (twice) from ethanol as colourless, small plates, m.p. 60-61°.

undepressed on admixture with authentic palmitic acid, m.p.60-62°.

Fraction (b). Identification of stearic acid. Isolation of the acid from this fraction after saponification gave a crystalline solid (after recrystallisation from ethanol), m.p.67-68.5°, undepressed on admixture with genuine stearic acid, m.p.68-69°.

Fraction (c). Isolation of the acid from this fraction after saponification gave a deep yellow crystalline solid, which was recrystallised from ethanol (charcoal), to give cerotic acid, m.p.73.5-77.5°. Admixture with cerotic acid m.p.82°, showed a depression of 1°, m.p.72.5-76.5°.

Isolation of the unsaturated acids from the lead salts. The filtrate, from the separation of the lead salts of the saturated acids, was concentrated to remove ethanol, and the residue extracted with light petroleum, (4 x 100ml.). The light petroleum extracts were bulked, washed with water, dilute hydrochloric acid (to decompose any lead salts still present), and again with water. The petrol was dried (sodium sulphate) and the solvent removed under reduced pressure to give 4.59g. of a pale brown oily residue.

Esterification of unsaturated acids. This was carried out as described for saturated acids, using methanol (100ml.) which contained sulphuric acid (2ml.).

Fractional distillation of the methyl esters. This was carried out as before in a semi-micro apparatus with a short fractionating column, and gave the fractions recorded in Table 8, (page 84).

Fraction (d). Identification of linoleic acid. The acid was extracted from the saponified ester of fraction (d) as a pale yellow oil which did not solidify at room temperature. It was dissolved in light petroleum (10ml.), and treated with a solution of bromine in light petroleum (2%) until bromine was in excess. The solution, on cooling in a refrigerator for 2 hours, deposited a very pale yellow solid, which was recrystallised from a mixture of ether and light petroleum (1-5) as colourless needles of tetrabromostearic acid, m.p.  $115^{\circ}$  with preliminary sintering at  $112-113^{\circ}$ . The crystals contained bromine and were undepressed ( $115^{\circ}$ ) on admixture with authentic tetrabromostearic acid from linoleic acid.

The mother liquors were concentrated in vacuo, when oily crystals were obtained, m.p. 25-40 . The mixture could not be resolved.

Fraction (e).    Identification of oleic acid.    The acid was extracted from fraction (e) after saponification, as a yellow oil which did not solidify at room temperature. A mixture of equal volumes of toluene and amyl alcohol (10ml.), 1 drop of phenolphthalein, and a slight excess of powdered barium hydroxide (slightly more than was necessary to neutralise the oleic acid), was heated on a boiling water-bath. The oily acid obtained (0.3g.) was added to the hot suspension, and the whole shaken for 5 minutes. The small amount of solid in suspension was allowed to settle, and the colourless supernatant liquid was poured off whilst still hot, and allowed to cool, when barium oleate separated as pale yellow prisms which were filtered, washed with toluene, and drained. The salt was recrystallised from 2.5 times its weight of a mixture of equal parts of amyl alcohol and toluene (a few drops of water being added), to give pure barium oleate, which was dried in vacuo. A suspension of the salt was shaken with water (10ml.) containing a slight excess of 10% solution of hydrochloric acid, for 4 hours. The solution was filtered, and the oily layer separated and shaken with dilute hydrochloric acid, and then with water until free from barium. The washed oleic acid was filtered through paper to remove traces of moisture, and dried over concentrated sulphuric acid in vacuo. Iodine value, found 91.68. (Calculated for  $C_{18}H_{34}O_2$ , 90.5).



The oil (0.2g.) was mixed with hydrogen peroxide (0.2g., 30%) and glacial acetic acid (0.6ml.), this mixture having previously been heated to  $85^{\circ}$  (1 hour), and cooled to  $25^{\circ}$  before mixing with the oil. The ensuing exothermic reaction was allowed to proceed, shaking occasionally. At  $71^{\circ}$  the mixture became homogeneous, and was allowed to cool slowly overnight. The solution was poured into hot water (5ml.), the aqueous layer being removed and rejected. The pale yellow oily layer was dissolved in N. caustic soda solution (2.5ml.), and heated for 2 hours on a steam bath. The hot solution was acidified with 6N. hydrochloric acid, cooled, and the solidified substance removed, washed with hot water (3ml.) which was slightly acid (HCl), cooled, water removed, and the solid recrystallised from aqueous ethanol (90%) as small plates of dihydrostearic acid m.p.  $90-91^{\circ}$ , undepressed on admixture with genuine dihydroxystearic acid. Neutralisation equivalent, found : 317.3; calculated for  $C_{18}H_{36}O_4$ : 316.

FRACTION III

This fraction was neutralised with a dilute solution of ammonia and evaporated to dryness under reduced pressure on a boiling water-bath. The pale-brown residue was mixed with anhydrous sodium sulphate (30g.), and this solid extracted by refluxing it in a Soxhlet with dry acetone for 6 hours. The acetone was cooled, filtered from salt, and evaporated to give a pale-brown residue (1.12g.). This was dissolved in water, decolourised with activated charcoal, filtered and the filtrate evaporated to give a colourless residue which was dissolved in dry ethanol, filtered from a trace of salt, and the filtrate evaporated to dryness. The residue was dried at  $100^{\circ}$  for 6 hours, to give a transparent liquid (0.88g.) which tasted of glycerol and gave the following colour reactions:-

- (1) Heated in a Bunsen flame on a borax-bead, it gave a green flame.
- (2) Heated with copper sulphate and sodium hydroxide solution the liquid was coloured blue.
- (3) Heated with potassium bisulphite, it gave off irritating vapours of acrolein. The vapours were passed through a tube into water, and the solution was found to reduce ammoniacal silver nitrate.

Glyceryl tri-p-nitrobenzoate. The residue (0.25g.) in

pyridine (4ml.), was mixed with a solution of p-nitrobenzoyl chloride (0.8g.) in pyridine (10ml.). The mixture was heated on a boiling water-bath for 30 minutes, cooled, and diluted with water (30ml.). Excess of a dilute solution of sodium hydroxide was added, and the mixture placed overnight in a refrigerator to give an oily solid which was filtered and recrystallised from aqueous acetone (90%), as small glistening plates of glyceryl-tri-p-nitrobenzoate, undepressed on admixture with authentic material, m.p. 191-192°. Nef<sup>(166)</sup> and Jaquemain and Maskovitz gave m.p. 192°<sup>(167)</sup>.

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PART II

THE SAPONINS

OF

CAULOPHYLLUM THALICTROIDES

## THEORETICAL

## INTRODUCTION

Caulophyllum thalictroides was first chemically investigated by Mayer<sup>(1)</sup>, who found a saponaceous principle and reported, but did not isolate, a colourless alkaloid. Ebert<sup>(2)</sup> also reported a saponin and an alkaloid, but he too failed to isolate the latter. This alkaloid was finally isolated, though not as a crystalline solid, by Lloyd<sup>(3)(4)</sup>, who called it caulophylline and did not determine its composition. He also isolated the saponin-like substance in a crystalline state, and found it to be a glucoside, which he called leontin. As a result of elementary analyses Trimble<sup>(5)</sup> assigned the formula  $C_{16}H_{26}O_5 \cdot H_2O$  to the saponin. Power and Salway<sup>(6)</sup> renamed the saponin caulosaponin, and reported a second saponin-like substance, present in much smaller proportion than caulosaponin, which they called caulophyllosaponin,  $C_{66}H_{104}O_{17}$ . The base was also isolated as a colourless crystalline alkaloid, which they showed to be methylcytisine  $C_{11}H_{13}ON_2(CH_3)$ .

Present interest in C. thalictroides arose through the identification of hederagenin as the sapogenin present in the closely related species, L. leontopetalum, Linn<sup>(7)</sup> (both Berberidaceae). Consideration of the melting points, specific rotations, physical and chemical properties, and analytical data of caulosapogenin and its derivatives, and



hederagenin and its derivatives, showed the two to be very similar (Table 1, page 209) and suggested that caulosapogenin could in fact be formulated as a triterpenoid  $C_{30}H_{48}O_4$ . An investigation was therefore undertaken to examine this hypothesis.

Caulosapogenin was first isolated by Power and Salway<sup>(6)</sup> from the roots and rhizomes of C. thalictroides, Linn. Michaux (Berberidaceae), by acid hydrolysis of an aqueous-ethanolic solution of caulosaponin. It was also isolated in the following year by Tutin and Clewer<sup>(8)</sup> in a somewhat similar manner from the flowering branches of Clematis vitalba, Linn. (Ranunculaceae). Power and Salway assigned to caulosapogenin the molecular formula  $C_{42}H_{66}O_6$  based upon elementary analyses and molecular weight determinations on the sapogenin and a number of derivatives, of which a di-, and tetra-acetate, a tetrabenzoate, and a methyl ether were described. Some doubt as to the correctness of this formulation was expressed by Tutin and Clewer based upon the analytical data of the sapogenin, the so-called methyl ether, and the benzoate. This is shown in Table 1, from which it will be seen that the analytical and other data found by Tutin and Clewer compare favourably with that found by the present author<sup>(11)</sup> for caulosapogenin from C. thalictroides, the caulosapogenin isolated being in every way identical with hederagenin,  $C_{30}H_{48}O_4$  from L. leontopetalum, and soap-nuts, Sapindus saponaria.

ISOLATION OF CAULOSAPONIN AND CAULOSAPOGENIN

An authentic sample of Caulophyllum B.P.C. 1934, consisting of the roots and rhizomes of C. thalictroides, Linn. was extracted by refluxing with boiling absolute ethanol, and the crude saponin precipitated by ether as described for leontosaponin. Recrystallisation of a small fraction of the saponin gave only one saponin, caulosaponin, as a white crystalline solid, as described by Power and Salway<sup>(6)</sup>. No attempt was made to fractionate the bulk of the crude saponins; instead they were hydrolysed directly with dilute acid to a colourless crystalline sapogenin,  $C_{30}H_{48}O_4$ , identical in every respect (melting point, mixed melting point, rotation and analysis) with a genuine sample of hederagenin. The presence of two hydroxyl groups in caulosapogenin was established by conversion to a diacetate, and by saponification of this acetate. The diacetate was identical in every respect with hederagenin diacetate. The proximity of the two hydroxyl groups to each other was shown by the formation of an isopropylidene derivative from the corresponding methyl ester. The sapogenin gave a yellow colour with tetranitromethane, and an ultra-violet absorption maximum at  $210\text{ m}\mu$  ( $\epsilon$ , 2,860) as for hederagenin, confirming the presence of a trisubstituted double bond. The presence of the carboxyl group was established by the formation of a

sodium salt and a methyl ester, the latter being identical with hederagenin methyl ester.

Power and Salway<sup>(6)</sup> also reported the isolation of a second saponin caulophyllosaponin from C. thalictroides (see Fig.1, page 208) which on hydrolysis gave a second sapogenin, caulophyllosapogenin  $C_{55}H_{88}O_9$ . The genins which they obtained on acid hydrolysis of the two saponins would appear to be identical (Table 2), since they had the same melting point, crystalline form, and physical properties, and gave the same colour reactions. The analytical figures were different, but this may have been due to impurities. The derivatives from both the sapogenins, viz. the acetates and methyl ethers also appear to be very similar (Table 2). Careful and repeated examination of the mother liquors remaining after the separation of caulosapogenin (hederagenin) in the present experiments, however, failed to reveal a second sapogenin, thus supporting the above theory that caulosapogenin and caulophyllosapogenin are identical.

Identification of caulosapogenin as hederagenin establishes the identity of most of the derivatives which were described by Power and Salway<sup>(6)</sup> and Tutin and Clewer<sup>(8)</sup>. Caulosapogenin tetrabenzoate from its melting point, specific rotation and analysis, must be regarded almost certainly as hederagenin dibenzoate. Caulosapogenin diacetate also would appear to be identical with hederagenin diacetate, from the

analytical data, the discrepancy in the melting point probably being due to the fact that hederagenin diacetate sinters at  $157-159^{\circ}$ , before melting at  $173-174^{\circ}$ ; the melting point of  $160-162^{\circ}$  reported by Power and Salway may in fact be the sintering point of the acetate. On the other hand the so-called caulosapogenin tetraacetate, described as an amorphous solid, melting at  $120^{\circ}$ , bears no relation to either the mono- or di-acetate of hederagenin, and the formation of this compound could not be repeated using the method of Power and Salway. The phenolic properties ascribed to the genin by Power and Salway<sup>(6)</sup> were based solely on the formation of a sodium salt from the diacetate. Tutin and Clewer<sup>(8)</sup> did not agree with the analytical figures of this salt as quoted by Power and Salway. Tutin and Clewer's analytical data for this salt is in excellent agreement with that for the monosodium salt of hederagenin diacetate. The methyl ether reported by both groups of authors is also similar to hederagenin methyl ester, in melting point, specific rotation and analytical data.

As already mentioned, two saponins were isolated from C. thalictroides by Power and Salway<sup>(6)</sup>. The first, caulosaponin, was insoluble in ether, whilst the second, caulophyllosaponin, present only in very small amounts, was sparingly soluble in ether. The method used by Power and Salway for the isolation of these two saponins is shown in

Fig.1. The present work has verified the presence of caulosaponin in the drug, but only one saponin has been isolated, and this saponin was insoluble in anhydrous ether. The mother liquors remaining after the isolation of caulosaponin were worked up to give a further yield of caulosaponin only. Colouring matter was removed from the saponin, in the purification process, by trituration with cold dry ethanol, and this ethanol extract was found to contain a small amount of saponin. It was also found that on shaking an aqueous solution of caulosaponin with ether, a small amount of saponin was soluble in the ether, but this saponin could be completely removed from the ether by thorough washing with water. The saponin extracted in the aqueous washings, and also the saponin in the colouring matter isolated above were chromatographed on paper as described for leontosaponin, along with an aqueous solution of caulosaponin. This showed that the saponins present in all three fractions were identical, having an  $R_F$  averaging 0.344, and that caulosaponin was homogeneous, only one spot being obtained which did not show any signs of tailing.

The method of isolating caulophyllosaponin as used by Power and Salway is interesting in that the authors state that "some difficulty was experienced in purifying this substance owing to the presence of small amounts of citrullol and caulosaponin, but after a process of fractional crystallisation from dilute ethanol, it was obtained in colourless

silky needles, melting and decomposing at 250-260°C. The substance was glucosidic and anhydrous." Thus caulosaponin, although reported by the authors to be insoluble in ether, was obviously soluble to the extent of interfering with the crystallisation of caulophyllosaponin. As already mentioned, it has been found that caulosaponin was soluble only in moist ether. Thus it is conceivable that Power and Salway may have experienced the same difficulty, and their caulophyllosaponin may in fact be caulosaponin contaminated with citrullol. The fact that caulophyllosaponin was obviously impure was apparent from the wide range (10°) of its melting point. The reported melting point of 250-260° should be compared with 254-255° for pure caulosaponin.

Analytical data reported for the two saponins (Table 2) were significantly different, but could possibly be accounted for by the fact that caulophyllosaponin was reported as anhydrous, whereas caulosaponin was hydrated; however the question of homogeneity of caulophyllosaponin is probably more significant. The melting point (160-161°) of caulosaponin acetate (Table 2) isolated in the present work was in reasonable agreement with that reported by Power and Salway for caulophyllosaponin acetate (155-160°). The acetate isolated in the present work, which melted at 160-161°, did so with preliminary sintering at 138-140°, and it may have been this sintering point which was quoted by the authors as

the melting point ( $135-140^{\circ}$ ) for caulosaponin acetate. With leontosaponin, it was found that even when an impure saponin was used in the preparation of an acetate, a pure acetate was obtained, and this may be cited as evidence that caulophyllosaponin is an impure form of caulosaponin. The specific rotation quoted for caulophyllosaponin ( $+32.3^{\circ}$ ) has not been observed, caulosaponin, isolated in the present work, being optically inactive. Power and Salway gave no rotation for caulosaponin.

According to Power and Salway, however, the two saponins gave different sugars on acid hydrolysis, glucose from caulosaponin, and arabinose from caulophyllosaponin. It has now been found that acid hydrolysis of caulosaponin using the method described by Power and Salway yields both glucose and arabinose, a fact which further supports the conclusion that caulophyllosaponin was merely an impure form of caulosaponin.

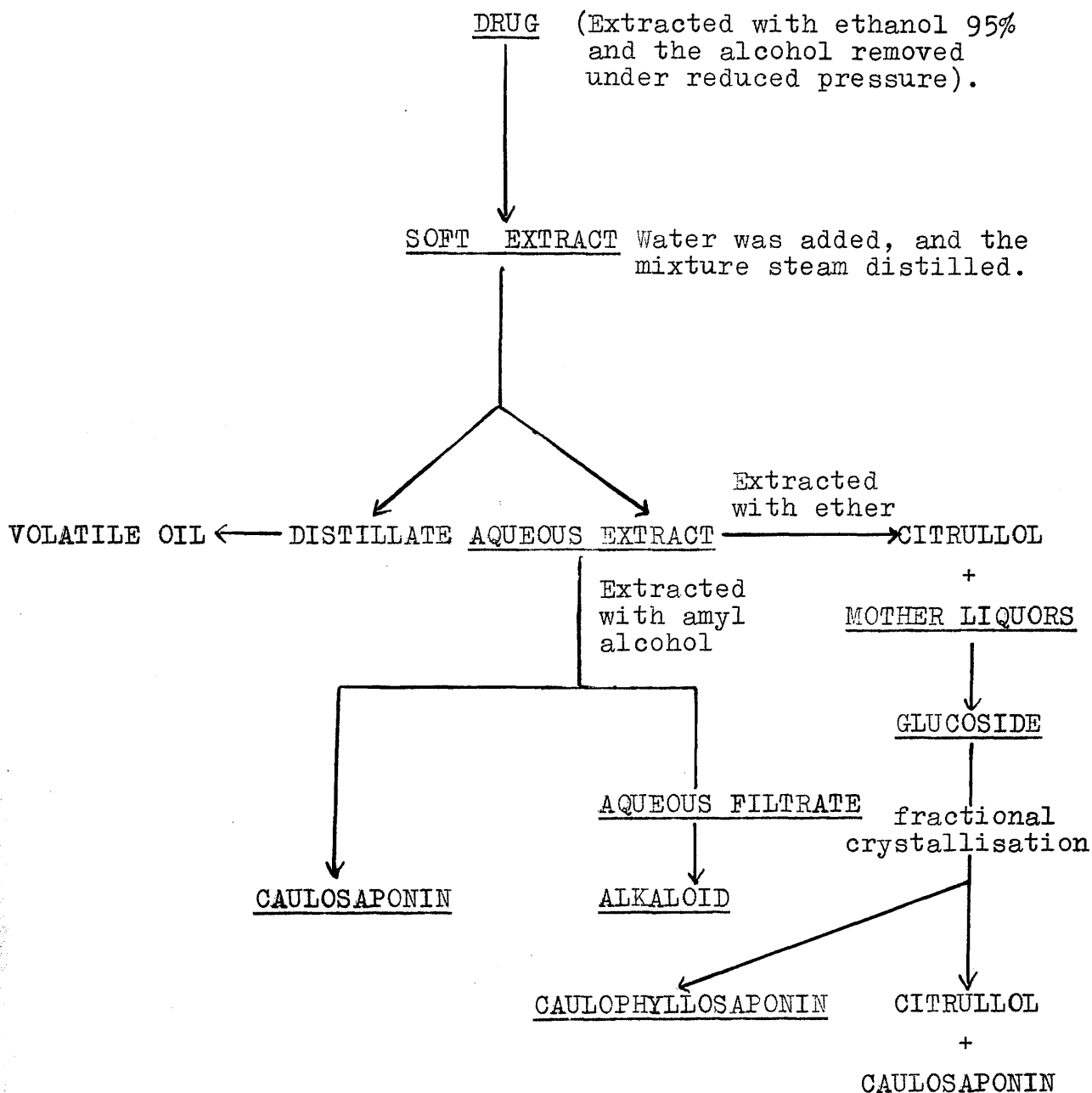


FIGURE 1.

Method used by Power and Salway to isolate Caulosaponin and Caulophyllosaponin.



Authors	Power and Salway (6)	Tutin and Clewer (8)	McShefferty and Stenlake (11)
Substance	CAULOSAPOGENIN.	CAULOSAPOGENIN.	CAULOSAPOGENIN = HEDERAGENIN
Source	<u>C. thalictroides</u>	<u>Clematis vitalba</u>	<u>C. thalictroides</u>
m.p.	315°	323°	Sinters 319°, melts 332-333°
Physical properties	Insoluble in water, benzene, chloroform. Sparingly soluble in ethanol, readily soluble in hot ethanol and aqueous ethanol.	-	As in column 2.
Colour reaction	Dissolved in acetic acid and chloroform, 2 drops of conc. sulphuric added, a rose-red colour was obtained.	-	As in column 2.
Analysis	C, 75.6, 75.7; H, 10.0, 10.1. C <sub>42</sub> H <sub>66</sub> O <sub>6</sub> requires: C, 75.7; H, 9.9%	Found: C, 75.8; H, 10.2. C <sub>42</sub> H <sub>66</sub> O <sub>6</sub> requires: C, 75.7; H, 9.9%	Found: C, 76.3; H, 10.3. Calculated for C <sub>30</sub> H <sub>48</sub> O <sub>4</sub> : C, 76.2; H, 10.2%
Substance Form and m.p.	CAULOSAPOGENIN TETRABENZOATE Prisms, 288° + 111°	CAULOSAPOGENIN TETRABENZOATE (1) Stout needles, 252° (2) Mainly prisms, 282° Not given	CAULOSAPOGENIN DIBENZOATE Prisms, 290-291° + 114° (at 20°)
Analysis	Found: C, 77.3; H, 7.9% C <sub>70</sub> H <sub>82</sub> O <sub>10</sub> requires: C, 77.6; H, 7.6%	Found: (1) C, 78.4; H, 8.3% (2) C, 77.4; H, 8.3%	Found: C, 77.4; H, 8.4. Calculated for C <sub>44</sub> H <sub>56</sub> O <sub>6</sub> : C, 77.6; H, 8.3%
Substance Form and m.p.	CAULOSAPOGENIN DIACETATE Needles, 160-162°	-	CAULOSAPOGENIN DIACETATE Needles, sinter at 157-159° melt at 173-174°
Analysis	Found: C, 73.4; H, 9.4. C <sub>46</sub> H <sub>70</sub> O <sub>8</sub> requires: C, 73.6; H, 9.3%	-	Found: C, 73.6; H, 9.6. Calculated for C <sub>34</sub> H <sub>52</sub> O <sub>6</sub> : C, 73.3; H, 9.4%
Substance Analysis	DIACETYLMONOSODIOCAULOSAPOGENIN Found: Na, 3.0% C <sub>46</sub> H <sub>69</sub> O <sub>8</sub> Na requires: Na, 3.0%	Found: Na, 3.7% Na, 3.0%	- C <sub>34</sub> H <sub>51</sub> O <sub>6</sub> Na requires: Na, 3.97%
Substance Form and m.p.	CAULOSAPOGENIN METHYL ETHER Needles, 235°	CAULOSAPOGENIN METHYL ETHER Needles, 229°	CAULOSAPOGENIN METHYL ETHER Needles, 236-237°
Analysis	+74.4° Found: C, 75.7; H, 10.3; OMe, 5.5% Tutin and Clewer found on methylation of Power and Salway's sample of caulosa-pogenin: C, 76.5; H, 10.5; OMe, 7.5%	+73.3° Found: C, 76.5, 76.4; H, 10.5, 10.5; OMe, 8.1, 7.8%	+76° (20° in chloroform) Found: C, 75.9; H, 10.5; OMe, 6.5% Calculated for C <sub>31</sub> H <sub>50</sub> O <sub>4</sub> : C, 75.9; H, 10.6; OMe, 6.4%

Table 1. Comparison of caulosa-pogenin isolated by different authors.

Authors	Power and Salway (6)			McShefferty and Stenlake (11)
Substance	CAULOSAPONIN.	CAULOPHYLLOSAPONIN.	CAULOPHYLLOSAPONIN.	CAULOSAPONIN.
Form and m.p.	Slender needles, 250-255°, (decomp.)	Silky needles, 250-260°, (decomp.)	Silky needles, 250-260°, (decomp.)	Micro-prisms, 254-255°, (decomp.)
Physical properties	Insoluble in water, ether, chloroform, and benzene. Sparingly soluble in cold ethanol, more readily soluble in hot ethanol.	Insoluble in water, sparingly soluble in ether, very soluble in ethanol.	Insoluble in water, sparingly soluble in ether, very soluble in ethanol.	Moderately soluble in water. Insoluble in dry ether, chloroform, and benzene. Sparingly soluble in cold ethanol, more readily soluble in hot ethanol.
$[\alpha]_D$	Not reported	+ 32.3° (ethanol)	+ 32.3° (ethanol)	+ 0° (ethanol)
Analysis	Found (anhydrous saponin); C <sub>64</sub> H <sub>108</sub> O <sub>17</sub> requires: C, 64.3; H, 8.7%	Found: C, 67.7, 67.8; H, 9.0, 9.3. C <sub>66</sub> H <sub>104</sub> O <sub>17</sub> requires: C, 67.8; H, 8.9%	Found: C, 67.7, 67.8; H, 9.0, 9.3. C <sub>66</sub> H <sub>104</sub> O <sub>17</sub> requires: C, 67.8; H, 8.9%	-
Substance	CAULOSAPONIN DECAACETATE	CAULOPHYLLOSAPONIN DECAACETATE	CAULOPHYLLOSAPONIN DECAACETATE	CAULOSAPONIN X-ACETATE
Form and m.p.	Amorphous, 135-140°	Amorphous, 150-160°	Amorphous, 150-160°	160-161°
Analysis	Found: C, 62.2; H, 7.6. C <sub>74</sub> H <sub>108</sub> O <sub>27</sub> requires: C, 62.2; H, 7.6%	Found: C, 64.4; H, 7.9. C <sub>86</sub> H <sub>124</sub> O <sub>27</sub> requires: C, 65.0; H, 7.8%	Found: C, 64.4; H, 7.9. C <sub>86</sub> H <sub>124</sub> O <sub>27</sub> requires: C, 65.0; H, 7.8%	-
Substance.	CAULOSAPOGENIN.	CAULOPHYLLOSAPOGENIN.	CAULOPHYLLOSAPOGENIN.	CAULOSAPOGENIN.
Form and m.p.	Rhombic, about 315°	Rhombic, about 315°	Rhombic, about 315°	Rhombic, sinters 319°, melts 332-333°
Analysis	Found: C, 75.6, 75.7; H, 10.0, 10.1. C <sub>42</sub> H <sub>66</sub> O <sub>6</sub> requires: C, 75.7; H, 9.9%	Found: C, 74.2, 74.0; H, 9.8, 9.9. C <sub>56</sub> H <sub>88</sub> O <sub>9</sub> requires: C, 74.3; H, 9.7%	Found: C, 74.2, 74.0; H, 9.8, 9.9. C <sub>56</sub> H <sub>88</sub> O <sub>9</sub> requires: C, 74.3; H, 9.7%	Found: C, 76.3; H, 10.3. Calculated for C <sub>30</sub> H <sub>48</sub> O <sub>4</sub> : C, 76.2; H, 10.2%
Substance	CAULOSAPOGENIN DIACETATE.	CAULOPHYLLOSAPOGENIN HEXAACETATE.	CAULOPHYLLOSAPOGENIN HEXAACETATE.	CAULOSAPOGENIN DIACETATE.
Form and m.p.	Needles, 160-162°	Amorphous, 160-162°	Amorphous, 160-162°	Needles, sinter 157-159°, melt 173-174°
Analysis	Found: C, 73.3; H, 9.4; C <sub>46</sub> H <sub>70</sub> O <sub>8</sub> requires: C, 73.6; H, 9.3%	Found: C, 70.2; H, 9.0. C <sub>68</sub> H <sub>100</sub> O <sub>15</sub> requires: C, 70.6; H, 8.7%	Found: C, 70.2; H, 9.0. C <sub>68</sub> H <sub>100</sub> O <sub>15</sub> requires: C, 70.6; H, 8.7%	Found: C, 73.6; H, 9.6. Calculated for C <sub>34</sub> H <sub>56</sub> O <sub>6</sub> : C, 73.3; H, 9.4%
M.W.	-	1130	1130	-
Substance	CAULOSAPOGENIN MONOMETHYL ETHER.	CAULOPHYLLOSAPOGENIN DIMETHYL ETHER.	CAULOPHYLLOSAPOGENIN DIMETHYL ETHER.	CAULOSAPOGENIN METHYL ESTER
Form and m.p.	Needles, 235°	Needles, 240-242°	Needles, 240-242°	Needles, 236-237°
$[\alpha]_D$	+ 74.4°	+ 43.6°	+ 43.6°	+ 76°
Analysis	Found: C, 75.7; H, 10.3; OMe, 5.5. C <sub>43</sub> H <sub>68</sub> O <sub>6</sub> requires: C, 75.9; H, 10.0; OMe, 4.6%	Found: C, 74.8; H, 10.1; OMe, 6.4. C <sub>58</sub> H <sub>92</sub> O <sub>9</sub> requires: C, 74.7; H, 9.9; OMe, 6.7%	Found: C, 74.8; H, 10.1; OMe, 6.4. C <sub>58</sub> H <sub>92</sub> O <sub>9</sub> requires: C, 74.7; H, 9.9; OMe, 6.7%	Found: C, 75.9; H, 10.5; OMe, 6.5. Calculated for C <sub>31</sub> H <sub>50</sub> O <sub>4</sub> : 75.9; H, 10.6; OMe, 6.4%
Sugars obtained on acid hydrolysis of saponin	Glucose	Arabinose	Arabinose	Glucose and arabinose

Table 2. Comparison of the two saponins, and the two sapogenins, and their derivatives

obtained by various authors.

## EXPERIMENTAL

Rotations were determined in 95% ethanol (unless otherwise stated), in a 1dm. tube. Ultra-violet absorption spectra were determined in absolute ethanol on a Hilger Uvispek photoelectric spectrophotometer.

Material. This was authentic *Caulophyllum* B.P.C. (1934) obtained through the usual commercial channels, and consisting of the dried roots and rhizomes of *Caulophyllum thalictroides*, Linn. Mich. (Berberidaceae).

#### Isolation of the Crude Saponin.

The drug (350g.) in No.80 powder was exhausted with boiling absolute ethanol (2l.). The ethanol extract was filtered whilst still hot, partially cooled, and the saponin precipitated as a dark yellow sticky mass with dry ether. The product, recrystallised from ethanol, gave the crude saponin (8.2g.) as a light tan amorphous solid, m.p.191-196° (decomp.). Treatment of the filtrate with ether as before, gave a further 3.0g. of crude saponin m.p.190-198° (decomp.). The saponin fractions were bulked and 1.2g. reserved for future examination.

#### Hydrolysis of Crude Saponin.

(a) The crude saponin (10g.) obtained above, was heated with ethanol (200ml.) and dilute hydrochloric acid (60ml.) under reflux for 5 hours. The gelatinous precipitate which

first formed, gradually crystallised, and was separated after cooling. It was dissolved in boiling ethanol, decolourised with charcoal, filtered, and recrystallised several times from boiling ethanol to constant m.p.  $332-333^{\circ}$ , with slight preliminary sintering at  $319^{\circ}$ .  $\epsilon$ , 2860 at  $210 \text{ m}\mu$ ,  $[\alpha]_D^{20} + 78^{\circ}$  (c, 0.1 in ethanol)  $[\alpha]_D^{20} + 79^{\circ}$  (c, 0.1 in pyridine). Jacobs<sup>(9)</sup> gives for hederagenin m.p.  $332-334^{\circ}$ ,  $[\alpha]_D^{20} + 81^{\circ}$  (c, 2.009 in pyridine). Mixed m.p. of sapogenin with authentic hederagenin,  $332-333^{\circ}$ . (Found : C, 76.3; H, 10.3%, equiv. 474.9. Calculated for  $\text{C}_{30}\text{H}_{48}\text{O}_4$  : C, 76.2; H, 10.2%, equiv. 472.7. The physical and analytical data found by Power and Salway<sup>(6)</sup> and Tutin and Clewer<sup>(8)</sup> for caulosapogenin and its derivatives are shown in Table 1.

The mother liquors obtained above were evaporated to dryness under reduced pressure, and the residue recrystallised to give more caulosapogenin m.p.  $332-333^{\circ}$ . No other solid was isolated. The charcoal was re-extracted with boiling ethanol (6 x 30ml.), when a further small yield of caulosapogenin only was obtained.

(b) The drug (725g.) was completely exhausted with boiling ethanol, and concentrated extracts precipitated with ether as before, to give 25.5g. of crude saponin m.p.  $201-215^{\circ}$  (decomp.). A portion of this saponin (5.2g.) was reserved for future examination. The remainder (20.3g.  $\equiv$  578g. of

root), was hydrolysed directly to yield 6.22g. of crude sapogenin which was purified as above to yield 5.808g. of caulosapogenin, m.p. 333-333.5°. The mother liquors, and charcoal, were again thoroughly examined, but in both cases, only further yields of caulosapogenin were obtained.

Caulosapogenin Diacetate. The sapogenin (0.5g.) was refluxed with acetic anhydride (5ml.) for 1 hour, the mixture poured into water, and the gum obtained recrystallised from aqueous ethanol (50%) to give stout needles melting at 173-174°, after preliminary sintering at 157-159°.  $[\alpha]_D^{20} + 64^\circ$  (c, 0.312). Jacobs<sup>(9)</sup> gives for hederagenin diacetate, m.p. 172-174°, with sintering at 156-159°,  $[\alpha]_D + 64^\circ$  (c, 1.0). Mixed m.p. with authentic hederagenin diacetate, 172-174°. (Found : C, 73.6; H, 9.6%, equiv. (by hydrolysis) 553. Calculated for  $C_{34}H_{52}O_6$  : C, 73.3; H, 9.4 per cent; equiv. 556.7).

Caulosapogenin Dibenzoate. The sapogenin (1g.) was refluxed with benzoyl chloride (1ml.) and pyridine (10ml.) for 1 hour on a boiling water-bath and the reaction mixture poured into a saturated aqueous solution of sodium bicarbonate. The gummy product was recrystallised several times from aqueous ethanol (60%) to give well-formed, colourless prisms, m.p. 290-291°,  $[\alpha]_D^{20} + 114^\circ$  (c, 0.112 in chloroform). Jacobs<sup>(9)</sup> gives for hederagenin dibenzoate, m.p. 290-291°

Mixed melting point with genuine hederagenin dibenzoate, 290-291°. (Found : C, 77.4; H, 8.4; Calculated for  $C_{44}H_{56}O_6$  : C, 77.6; H, 8.3 per cent).

Caulosapogenin Methyl Ester. The sapogenin (1.005g.) was dissolved in 100ml. of an ether solution containing excess of diazomethane, shaking vigorously for  $\frac{1}{2}$  hour. On evaporation to dryness, the methyl ester was obtained, and this was recrystallised several times from aqueous ethanol (50%) to give colourless needles, m.p. 236-237°.  $[\alpha]_D^{20} + 75.96$  (c, 0.498 in chloroform). Jacobs<sup>(9)</sup> gives for hederagenin methyl ester, m.p. 238-240°  $[\alpha]_D + 76^\circ$  in chloroform. Mixed melting point with authentic hederagenin methyl ester, 235-236.5°. (Found : C, 75.9; H, 10.5. Calculated for  $C_{31}H_{50}O_4$ , C, 75.9; H, 10.5 per cent).

Caulosapogenin Methyl Ester Diacetate. The methyl ester (0.287g.) was refluxed with acetic anhydride (5ml.) as before, to give stout colourless needles, after three recrystallisations from aqueous ethanol (50%), m.p. 192-193°.  $[\alpha]_D^{20} + 63^\circ$  (c, 0.564). Van der Haar<sup>(10)</sup> gives for hederagenin methyl ester diacetate m.p. 193°  $[\alpha]_D + 62^\circ$  (ethanol). Mixed m.p. with hederagenin methyl ester diacetate, 192-193°. (Found : C, 74.0; H, 9.7. Calculated for  $C_{35}H_{54}O_6$  : C, 73.6; H, 9.5%).

Acetonyl Caulosapogenin Methyl Ester. A solution of the methyl ester (0.30g.) in acetone (4ml.) with two drops of concentrated hydrochloric acid deposited platelets of the acetonyl caulosapogenin methyl ester. They were recrystallised several times from ethanol, giving lustrous leaflets, m.p. 250-251° alone, or mixed with genuine acetonyl hederagenin methyl ester. (Found : C, 77.5; H, 10.3. Calculated for  $C_{34}H_{54}O_4$  : C, 77.5; H, 10.3%).

Purification of the crude saponin fractions.

The crude saponin fractions were bulked (6.4g.), and redissolved in boiling ethanol (500ml.), the solution filtered whilst hot, cooled, and precipitated with dry ether as before. This process was repeated thrice to give a pale yellow amorphous solid, m.p. 225-235°. The solid was then triturated with successive small quantities (10ml.) of cold anhydrous ethanol, until no more yellow colour was removed. The pale buff saponin, m.p. 249-253° was then recrystallised from boiling ethanol to give colourless, micro-prisms of constant m.p. 254-255° (decomp.). The mother-liquors still contained some saponin and were bulked, the ethanol removed, and the residue treated with cold anhydrous ethanol as before to give a further small amount of caulosaponin, m.p. 253.5 - 254.5° (decomp.). The yellow solution of colouring matter soluble in cold anhydrous ethanol gave a faint reaction



(concentrated sulphuric acid) for saponin. This solution (a) was retained for chromatographic examination. Pure caulosaponin (0.5g.) in water (50ml.) was now repeatedly extracted with ether (10 x 25ml.). A small amount of saponin was extracted by the ether. The bulked ether extracts were washed well with water (20 x 10ml.) and the washings concentrated and reserved, solution (b), for chromatographic examination. The washed ether was dried (sodium sulphate), and the solvent removed. No saponin was obtained. The original aqueous solution of caulosaponin was freed from ether, and this solution, (c), chromatographed on paper along with solutions (a) and (b) by the method of upward development for 24 hours, using the same solvent system and spray reagent as used for leontosaponin, (page 39).

Solution (a) gave a faint yellow, small round spot, average  $R_F$  0.344.

Solution (b) gave a yellow, small round spot, average  $R_F$  0.343.

Solution (c) gave a yellow, round compact spot, average  $R_F$  0.346.

Caulosaponin Acetate. Caulosaponin (0.5g.) was refluxed with acetic anhydride (10ml.) and sodium acetate (0.5g.) on a water-bath until solution was effected, and then for a further two hours. The reaction mixture was poured on to

ice, and the sticky solid extracted with ether, the ether bulked, washed with water, dried (sodium sulphate), and evaporated to give a pale yellow solid which was recrystallised from ethanol (60%) as colourless, small needles, m.p.  $160-161^{\circ}$ , with preliminary sintering at  $138-140^{\circ}$ .

Examination of the sugar solution obtained on acid hydrolysis of caulosaponin. The sugar solution was neutralised with caustic soda solution (20%) and decolourised with charcoal as before. The filtered colourless solution gave positive reactions in Molisch's, Benedict's, Fehling's and Barfoed's tests, and gave positive tests for pentose sugars, with both the Aniline and Bial's reagent.

Osazone formation. The sugar solution (5ml.) was heated with phenylhydrazine hydrochloride (4g.) and anhydrous sodium acetate (0.6g.) on a boiling water-bath. After 15 minutes a crystalline solid separated and was rapidly filtered off, when it was found to be identical, (m.p.  $200-206^{\circ}$ , and crystalline form, clusters of long needles), with glucosazone. The filtrate, heated for a further 10 minutes gave a small amount of a tangled mass of crystals which contained small amounts of crystals which were indicative of arabinosazone, and celloliosazone, respectively.

Treatment with lead acetate. The sugar solution (4ml.) was boiled with a small amount of lead acetate, cooled, dilute

solution of ammonia added, and the solution reboiled, when a salmon pink precipitate was obtained.

Paper chromatography of the sugar solution.

This was carried out exactly as for the sugars obtained from leontosaponin (page 118), the solution being compared with standard (1%) solutions of D-glucose and L-arabinose. Three spots were obtained, the first, spot (a), was a faint, round, yellow spot, average  $R_F$ , 0.343. (Caulosaponin had  $R_F$ , 0.346). The second spot (b), a small, cherry-red spot with some tailing, had an average  $R_F$ , 0.252. (L-Arabinose had  $R_F$ , 0.258), and the third spot, (c), a large, irregular, yellowish-brown spot, had an  $R_F$ , 0.213. (D-Glucose had  $R_F$ , 0.221). Comparison of the areas of spots (c) and (b) showed the former to be approximately four times as great as spot (b).

- [illegible]

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PRELIMINARY PHARMACOLOGICAL INVESTIGATIONS

A preliminary examination by Dr.G.Brownlee<sup>(1)</sup> of King's College, London, of the powdered dry root-tuber of Leontice leontopetalum, showed that the crude preparation, obtained by maceration of the drug with saline, when applied in the equivalent of 100mg. to rabbit duodenum contained a powerful spasmogenic principle which was not acetylcholine-like. The crude material, however, killed the gut. The crude preparation was very toxic when given intravenously to the cat; single doses as little as 80mg. equivalent, produced profound central stimulating action in the barbiturate anaesthetised animal (one observation). Acute generalised muscular twitching implicated the spinal cord, but there was also a fall in blood pressure, which was immediate. This did not seem to be histamine, or histamine-like as it seems to be seen as a contraction of the ileum, in the presence of mepyramine.

A second preliminary examination by Dr.Brownlee<sup>(2)</sup>, of crude alkaloid extracts, and of crude saponin isolated from L. leontopetalum was carried out. Three samples were supplied :

- (A) A solution in water of the crude hydrochloride of the crude chloroform-soluble alkaloids, 50mg./ml.
- (B) A solution in water of the crude base chloride obtained

from the mercuric chloride complex of the water-soluble alkaloid, 50mg./ml.

(C) Crude saponin, m.p. 215-230° (decomp.).

Dr. Brownlee reported that solutions (A) and (B) were similar in action and probably contained the same substance, solution (B) being more potent than solution (A). The active material in these solutions had the activity of a central nervous depressant with a substantial action on the spiral cord. At no time was there complete paralysis. Death, when it occurred, was due to respiratory failure. The saponin was surprisingly non-toxic, had little lysogenic activity, and showed two actions:-

- (i) a stimulant of central nervous activity, and
- (ii) a depressant of central nervous activity in large doses.

A further investigation was carried out by Mr. J.L. Paterson<sup>(3)</sup> of this department, on the pure substances isolated from the drug. Petaline chloride was found to act as a central nervous depressant on intravenous administration to both mouse and rabbit. Death was due to respiratory failure. It also showed anti-acetylcholine activity on isolated skeletal frog muscle, the relative potency of petaline chloride to gallamine tri-ethiodide being 0.643 : 1.

The effect of leonticine in mouse appeared to be similar to that of petaline chloride, although it was significantly



less potent, and bulbar paralysis was preceded by active clonic spasms. Again death was due to respiratory failure. Both petaline chloride and leonticine were very toxic to both mouse and rabbit, the mean Lethal Dose of Petaline Chloride for the rabbit being 15.6mg./Kg. The Therapeutic Ratio was also very low, approximately 1.5, and this substantiated Dr. Ford Robertson's report of the toxicity of L. leontopetalum.

No pharmacological results are yet available on leontamine.

Leontosaponin had little observed lysogenic activity, and the pure substance was not tested on mouse or rabbit.

Ferguson and Edwards<sup>(4)</sup> reported that caullosaponin, the saponin from Caulophyllum thalictroides, had oxytocic activity, but careful and repeated examination of both leontosaponin and caullosaponin, failed to reveal any evidence of oxytocic activity.

1. *Journal of Communication* 1954 51:3-4:100-101.

2. *Journal of Communication* 1954 51:3-4:100-101.

3. *Journal of Communication* 1954 51:3-4:100-101.

4. *Journal of Communication* 1954 51:3-4:100-101.

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- (1) Personal communication from Dr.G.Brownlee.
- (2) Personal communication from Dr.G.Brownlee.
- (3) McShefferty, Nelson, Paterson, Stenlake and Todd,  
J.Pharm.Pharmacol., 1956, 1117.
- (4) Ferguson and Edwards, J.Amer.Pharm.Assoc., 1954, 43, 16.